2019

Characterization and Analysis of Proteins Secreted by the Mutant Pichia pastoris Strain, bgs13

Christopher Alan Naranjo
University of the Pacific, c_naranjo@u.pacific.edu

CHARACTERIZATION AND ANALYSIS OF PROTEINS SECRETED BY THE MUTANT *PICHIA PASTORIS* STRAIN, *BGS13*

by

Christopher A. Naranjo

A Thesis Submitted to the

Graduate School

In Partial Fulfillment of the

Requirements for the Degree of

Master of Science

College of the Pacific
Biological Sciences

University of the Pacific
Stockton, California

2019

CHARACTERIZATION AND ANALYSIS OF PROTEINS SECRETED BY THE MUTANT *PICHIA PASTORIS* STRAIN, *BGS13*

By

Christopher A. Naranjo

APPROVED BY

Thesis Advisor: Geoffrey Lin-Cereghino, Ph.D.

Committee Member: Craig Vierra, Ph.D.

Committee Member: Douglas Risser, Ph.D.

Department Chair: Lisa Wrischnik, Ph.D.

Dean of Graduate School: Thomas Naehr, Ph.D

ACKNOWLEDGEMENTS

From our inception, we are propelled through a one-way trip into the unknown upon the arrow of time, like the feathers that compose the fletching at its rear. For me, this path proved to be an adventurous one, a welcomed challenge. The unyielding path compelled me to develop means of adapting to change. Change is always certain, and always approaching. Finding these methods of processing change proved to be an endeavor just as difficult as change itself. However, the sources of support and guidance around me – family, friends, knowledge, wisdom, and love – provided the means to overcome life's challenges.

To my family, I am grateful for all the love and support I have received throughout the years. Since my childhood, they taught me respect, integrity, patience, and perseverance. To my father, thank you for igniting the spark of curiosity into the world of science and technology that allowed me to reach the place where I am today. To my mother, thank you for being the most loving and caring person in my life; you will forever be a source of inspiration on how to treat others with compassion and love. To my brother, aunt, my grandparents, and the rest of the family, thank you for always supporting me during my studies and encouraging me to always do my best – in and out of school. The family is the anchor for every individual: unmoving, always there when you need it. As it grows and changes with time, we will always learn with each other. I hold my family as a boundless source of love that I can only hope to reciprocate with due time.

To my close friends, thank you for all of the good times we have spent together. We have had countless times filled with uncontrolled laughter, thought-provoking discussions, and

enough adventurous experiences that will allow us to reminisce by starting with “Remember that one time…” for the remainder of our lives. I hope we have learned from each other during our time, and that we may use those experiences in the future to find joy and better ourselves.

I cannot begin to express my appreciation towards my teachers, professors, and mentors that have shared knowledge with me and countless other students throughout the years. From those who taught me in kindergarten to the professors who led graduate-level courses, you have taken a part in shaping my mind, experiences, and outlook throughout my journey in academia. You have shared endless amounts of knowledge to me and many other students, and even revealed bits of wisdom when deemed necessary. For all of this and more, I thank you. I hope that you can always regard your role as a teacher as an indispensable part of society that has an unfathomably lasting impact on the world.

Change is inevitable, but that does not mean it has to prove formidable. The sources of love, wisdom, support, and inspiration that surround us must be recognized in full. Through this, one can experience their ride on the arrow of time with ease, elation and with little worry.

“Then as it was, then again it will be

And though the course may change sometimes

Rivers always reach the sea

Blind stars of fortune, each have several rays

On the wings of maybe, down in birds of prey

Kind of makes me feel sometimes, didn't have to grow

But as the eagle leaves the nest, it's got so far to go…”

From *Ten Years Gone*, by Led Zeppelin. Songwriters: Jimmy Page, Robert Plant

CHARACTERIZATION AND ANALYSIS OF PROTEINS SECRETED BY THE MUTANT *PICHIA PASTORIS* STRAIN, *BGS13*

Abstract

By Christopher Naranjo

University of the Pacific

2019

The methylotrophic yeast *Pichia pastoris* has been utilized for heterologous protein expression for research, clinical, and industrial purposes to produce thousands of recombinant protein products. Because *P. pastoris* secretes very few of its own proteins, the exported recombinant protein is the major polypeptide in the extracellular medium, making purification relatively easy. Unfortunately, a disadvantage to the programmed export is that some recombinant proteins intended for secretion are retained within the cell and may be subsequently degraded. A mutant strain isolated in our lab, containing a pREMI-derived disruption of the *BGS13* gene, has displayed elevated levels of secretion for a variety of reporter proteins. The wild-type protein expressed from this gene shares homology with the *S. cerevisiae* Pkc1 protein. It has been elucidated that the Bgs13 protein plays a crucial role in the *P. pastoris* secretory network, but its specific mode(s) of action is currently unclear.

To reveal the differences in the secretion mechanism and cell wall integrity between wild-type *P. pastoris* and the *bgs13* strain, we identified and characterized differential protein

populations found in the extracellular medium and those extracted from the cell wall. The proteomic approach revealed that the *bgs13* strain released an increased array of normally secreted proteins, endogenous proteins, and Endoplasmic Reticulum(ER)-resident chaperones. The role of the Bgs13 protein in the Cell Wall Integrity pathway was further investigated by an analysis of the relative cell wall porosity that indicated comparable porosity between the wild-type and *bgs13* strains. Because ER-resident chaperones were found to be released in higher abundance by the *bgs13* strain, intracellular protein disulfide isomerase levels were analyzed, but no major differences between the two strains were detected. Nonetheless, this result points to a possible occurrence of an unorthodox mode of protein sorting in the *bgs13* strain that leads to enhanced release of proteins and cell wall structural changes. This research explicated, in part, the underlying mechanisms of enhanced secretion of the *bgs13* strain.

TABLE OF CONTENTS

LIST OF TABLES

Table

LIST OF FIGURES

Figure

Chapter 1: Introduction

Heterologous Protein Expression

It is often viewed that the molecular workhorses of the cell are proteins, which are polymer chains made up of 20 possible amino acids arranged in different orders and lengths. Proteins have a wide range of functions in the biological realm. Some compose the architecture of the cell's structure and others can even come together to form a tubular protein complex that is capable of degrading other proteins to pieces as they pass through.

Differences in the primary amino acid sequence gives rise to unique proteins. The primary sequence of a protein's amino acid chain is the major determining factor of how the amino acid chain that composes the protein will fold in three-dimensional space. The primary amino acid sequence of a protein is determined by the nucleotide sequence of its respective gene, which follows the genetic code (Crick et al., 1961). The gene's corresponding DNA sequence is then transcribed into mRNA that will then be used as a template for protein synthesis via the ribosome in a process known as 'translation' (Bossi and Roth, 1980). Protein expression refers to the synthesis, modification, and regulation of proteins in biological systems. Major functions of proteins in cells include, but are most certainly not limited to, roles in metabolism, cellular structure and support, and regulation of gene transcription. Like the other macromolecules, proteins play essential roles in all domains of life by participating in all compartments and functions of a cell. To elucidate the roles and effects of proteins in biological processes, they must be systematically expressed and purified. Biological research of proteins encompasses both the study of the protein itself as well as the experimental techniques used for successful protein expression.

In order to produce a particular protein of interest, living cells and their natural cellular translational machinery must be utilized for this controlled expression. These cells can be considered to operate as "factories" that will initiate expression of protein that is derived from a known, given DNA sequence that encodes a gene - typically distinctive from what is naturally found in the specific host cell type. Species or cell types used for this form of protein expression are often referred to as expression systems.

Due to the relative ease of DNA synthesis and design, it is an essential tool for much of protein-based biological research: DNA templates can be incorporated and used by biologically active cells to heterologously express 'recombinant' protein. Recombinant protein expression is not only used to study proteins but has become one of the most prominent methods of synthesizing large-scale quantities of heterologous protein. The increased scale of production is typically prepared for commercial, clinical, and research purposes. Over the past few decades, heterologous protein expression has become one of the primary means to produce therapeutic and pharmaceutical proteins (Chen, 2012; Wurm, 2004; Gecchele et al., 2015; De Schutter et al., 2009).

The origin of the host cell type being used, or of the protein being studied, is a crucial factor in forming the recombinant protein expression system that will be used. Because prokaryotic and eukaryotic cells have vastly different cellular compositions and biological mechanisms (Kozak, 1999), they will give rise to different expression systems to be utilized for recombinant protein expression. *Escherichia coli,* with its extensively characterized genetics, is a widely used host organism for prokaryotic recombinant expression (Baneyx, 1999; Baneyx and Mujacic, 2004). Regarding eukaryotic heterologous protein expression, there is an array of host organisms than can be utilized.

Mammalian cells have proved useful in generating correctly folded, functional therapeutic proteins for treatment in humans due to their native cellular machinery. However, their innate complexity leads to challenges that must be overcome during recombinant protein cultivation from mammalian cells (Wurm, 2004). A relatively simple single-celled eukaryote that is widely used for heterologous protein production is the budding yeast (Cherry et al., 2012). The yeast's compartmentalized eukaryotic organelles allow a multitude of advantages over simpler prokaryotic systems including, but not limited to, post-translational modifications, polypeptide folding mechanisms, and regulation of proteins to achieve native biological activity. This includes disulfide bond formation, N-linked and O-linked glycosylation of amino acid residue (Lin-Cereghino and Cregg, 2000), and proteolytic maturation of proteins. Their unicellular nature allows for relatively rapid growth and genetic manipulation that is needed to optimally take advantage of the budding yeasts as a recombinant expression systems. Their ability to secrete proteins can also be considered as a valuable initial step of purification, which eliminates the need to account for cell lysis and any subsequent impurities that arise. Although *Saccharomyces cerevisiae* is the most used and characterized species of budding yeast, it carries certain disadvantages regarding its use as a recombinant expression system (Buckholz and Gleeson, 1991). Our focus is on an ascomycetous yeast that is at the forefront of eukaryotic recombinant protein expression: *Pichia pastoris*.

Pichia pastoris: **A Recombinant Expression System**

Since the 20th century, yeasts have been characterized and used for the purpose of recombinant protein expression. In the late 1960s, it was first described that certain yeasts had the capability to sufficiently grow on medium that utilized methanol as the only carbon source (Ogata et al., 1969). While *P. pastoris* was a species originally utilized to produce single-cell

protein animal feed, this application showcased the methylotrophic single-celled eukaryote's potential as a recombinant host organism. The foundation of *P. pastoris* as a recombinant protein expression lies upon some uniquely activated enzymes that are expressed in the methanol utilization pathway (Cregg et al., 1987; Lin-Cereghino and Cregg, 2000). When grown on methanol-containing medium, alcohol oxidase (AOX) is the first enzyme to encounter methanol in the peroxisome, catabolizing it into formaldehyde and hydrogen peroxide. From there onward lies more enzymatic activity that is part of the utilization pathway, including assimilation of formaldehyde to produce cellular constituents and energy for the cells growing on methanol (Lin-Cereghino and Cregg, 2000). Alcohol oxidase is present at large quantities when expression is induced on methanol but has undetectable levels when grown on other carbon sources. When expressed, it can account for up to 35% of the total protein produced in the *P. pastoris* cell (Sreekrishna et al., 1997). *AOX1* and *AOX2* are two genes that encode the AOX enzyme, but *AOX1* has been shown to be the primary gene that facilitates methanol oxidation (Cregg et al., 1989). Expression of the *AOX1* gene is transcriptionally regulated; its promoter element is repressed/derepressed by other carbon sources such as glycerol or glucose, and the presence of methanol induces *AOX1* derepression and expression (Tschopp et al., 1987).

The genetic techniques required to convert *P. pastoris* into an expression system include traditional standard molecular biology techniques such as DNA-mediated cloning and transformation via electroporation. Homologous recombination between genomic DNA and artificially introduced DNA is also utilized by *P. pastoris*; a form of homologous recombination arises when cleavage of an introduced DNA vector produces a sequence shared with the host genome that leads to integration of the vector into the genomic locus (Lin-Cereghino and Cregg, 2000). When compared to the introduction of centromeric vectors, integration into the genome

increases the stability of expression strains. The typical workflow of heterologous expression is composed of the following: Insertion of the foreign gene of interest into an expression vector, introduction of the vector into *P. pastoris*, screening for potential expression strains, selection/isolation of the strain(s) of interest, large-scale expression of the gene product, and final purification of the recombinant protein. Most *P. pastoris* strains that are used for recombinant expression are selected for successful transformation with vectors that contain an appropriate selectable marker (Lin-Cereghino and Cregg, 2000). Vectors used for foreign gene expression are usually *E. coli/P. pastoris* shuttle vectors. They contain an expression cassette composed of the 5' *AOX1* promoter sequences and a second *AOX1* fragment needed for transcriptional termination (Cregg et al., 1989). A multiple cloning site for the introduction of the gene of interest typically lies between these two *AOX1* sequences. These features mark *P. pastoris* as an exceptional choice as a recombinant expression system for the production of heterologous proteins.

The most attractive characteristic of *P. pastoris* that makes it preferred over other budding yeasts as a recombinant expression system is its disinclination to perform anaerobic fermentation. Its preference for aerobic growth enables *P. pastoris* to be grown in a fermentor (also known as a bioreactor) to achieve high cell-density cultures without producing toxic levels of ethanol. The ability of *P. pastoris* to be cultured in a shaker flask or in a fermentor allows for different scales of growth needed for a variety of applications. For example, small-scale research purposes might induce growth in shake flasks, whereas industrial production of a recombinant therapeutic will be done under large-scale fermentation conditions. A high cell density achieved from fermentation conditions usually leads to high levels of recombinant expression. With growth medium being solely composed of defined substances, void of any possibly toxins or

pyrogens, it further represents it as a compatible expression system to produce human pharmaceuticals (Lin-Cereghino and Cregg, 2000). *P. pastoris* has been used in the biotechnology industry to produce a variety of protein therapeutics for clinical, industrial, and research purposes, such as antigenic vaccines and monoclonal antibodies (Buckholz and Gleeson, 1991; Cregg et al., 1987; Li et al., 2006; Lin-Cereghino and Cregg, 2000).

The Secretory Network

In order to illuminate the mechanisms behind secretion, extensive characterization of the cellular constituents of *P. pastoris* has been performed. This primarily includes the secretory network as well as cell wall integrity.

The eukaryotic secretory network encompasses the endoplasmic reticulum (ER), Golgi apparatus, the vesicles that participate in transport, and the cell membrane. Compared to the cytosol, these organelles possess oxidative environments that are needed to synthesize and transport these proteins. The secretory network is a cellular pathway to shuttle proteins to their final destination within or out of the cell. As ribosomes synthesize proteins intended for secretion, detection of a signal peptide sequence (Lin-Cereghino and Cregg, 2000) directs co-translational binding to the ER and subsequent completion of translation within the lumen (von Heijne, 1990). Within the ER, proteins intended for secretion are correctly folded and modified by enzymes. These include protein disulfide isomerase (PDI) (Wilkinson and Gilbert, 2004; Li et al., 2010) that cross-links specific cysteine residues and oligosaccharyltransferases that performs N-linked glycosylation (Kelleher and Gilmore, 2006).

One of the primary functions of the ER is to ensure that there is quality control of proper protein folding prior to anterograde movement through the secretory pathway (Patil and Walter,

2001). Unfortunately, accumulation of misfolded proteins within the ER lumen can occur; this form of cellular stress from within the endoplasmic reticulum must be considered when conducting heterologous protein expression. Proteins must be properly folded with high fidelity in order to be ready for secretion out of the cell. When this does not occur, the Unfolded Protein Response (UPR) is triggered. The UPR is a set of signal transduction pathways that, once activated, can alleviate ER stress by attempting to maintain homeostasis (Patil and Walter, 2001; Ron and Walter, 2007; Ron and Walter, 2011). UPR activation leads to expansion of the ER membrane, as well as upregulated production of necessary protein-folding machinery in order to accommodate the high volume of proteins. In most high-order eukaryotes, three unique branches of signal transduction that compose the UPR work in parallel with their own mode of action (Ron and Walter, 2011). However, only one of the three branches of UPR signaling works solely in yeasts, including *Pichia pastoris*.

The highly conserved IRE1 (inositol requiring enzyme 1) branch of the UPR is the target of interest when considering the ER stress exerted on yeast cells during recombinant expression; in yeast, IRE1 activity induces the entirety of the UPR gene expression program (Ron and Walter, 2011). The transmembrane serine-threonineF kinase Ire1p acts as a sensor for ER stress. Unfolded protein accumulation in the ER leads to Ire1p oligomerization, and subsequent autophosphorylation leads to activation of its endonuclease activity. The endonuclease Ire1p then cuts *HAC1* mRNA at two sites to remove an intron and the two exons are joined by Rlg1p. Non-spliced "uninduced" *HAC1* mRNA is not translated. However, the spliced "induced" *HAC1* mRNA encodes Hac1p that is a transcriptional activator of UPR target genes (Patil and Walter, 2001). Hac1p controls the expression of several hundred genes (Delic et al., 2013). One of these downstream targets includes the ER-resident foldase protein disulfide-isomerase. This ER

chaperone aids newly produced proteins in achieving correct disulfide linkages. UPR activation upon recombinant protein expression provides another facet to study in order to optimize heterologous protein expression in *P. pastoris.* Having the proper three-dimensional structure is essential for anterograde movement toward the next organelle in the secretory network - the Golgi apparatus.

The Golgi is the site for protein maturation and sorting. Anterograde and retrograde transport of proteins between the ER and the Golgi apparatus is facilitated by highly dynamic membrane-bound transport vesicles (Kuehn et al., 1998). Proteins synthesized in the ER that are intended for secretion enter the early compartment of the Golgi apparatus, *cis* cisternae, undergo various post-translational modifications as it moves through the Golgi, and exit through the *trans* cisternae. The Golgi complex is a dynamic, non-contiguous organelle in which anterograde cargo vesicles from the ER either fuse together or with the Golgi (Lodish et al., 2008). The cisternae that compose the Golgi apparatus are flattened membrane stacks. The various parts of the Golgi have enzymes for specific post-translational modifications that are applied in a retrograde manner during the proteins' migration through the Golgi (Jackson, 2009). Despite two models being proposed for transport through the Golgi (one proposes vesicular transport, yet another suggests cisternal progression and maturation), the cisternae of the yeast Golgi dynamically change the resident membrane proteins from the early *cis* to late *trans* composition over time (Jackson, 2009; Matsuura-Tokita et al., 2006). The *trans* cisternae is the next site for proteins that are intended for secretion and is where vesicle formation for delivery to the plasma membrane occurs. One possibility is direct exocytosis of the cargo-containing vesicles into the extracellular space for continuously secreted proteins. However, if a protein is not ready to be secreted and must be stored within the cell, it will be packed into secretory vesicles in

preparation for later exocytosis. For budding yeast species such as *P. pastoris*, secreted proteins traverse through the periplasmic space and cell wall prior to exit into the extracellular space.

Yeast Cell Wall Integrity

The fungal cell wall is a structurally durable, yet dynamic, cellular structure. It acts as a means of osmoregulation, can sense extracellular stress, detect changes in cell shape, and plays a critical role in morphogenesis and remodeling (Levin, 2005; Jung and Levin, 1999; Levin, 2011).

The cell wall is a four-layered structure, and it is composed of four main structural components: ß-1,3 glucan, ß-1,6 glucan, chitin (a-ß- 1,4 N-acetylglucosamine polymer), and mannoproteins (a type of glycoprotein that contains mannose as a large portion of its weight). The inner layer is composed primarily of glucan polymers and chitin, rendering it exceptionally durable and elastic as the cell wall core (Levin, 2011). The outer layers, facing the periplasmic space and extracellular space, are matrices of mannoprotein that are linked to inner glucan chains (Levin, 2011; Kollár et al., 1997; Lipke and Ovalle, 1998). Because the cell wall is the first line of encounter with the extracellular environment, cell wall integrity (CWI) is a proxy for potential cell stress.

The cell wall integrity pathway is employed to manage cell wall perturbations and potential intracellular effects; this can occur naturally during normal growth or due to environmental changes. The CWI pathway has been extensively studied in *Saccharomyces cerevisiae*. Cell wall stress is sensed at the plasma membrane by transmembrane surface sensor proteins (i.e. *S. cerevisiae's* Sig1p and Mid2p) that are coupled to the small GTPases, such as Rho1p in *S cerevisiae*. In this example, Rho1p acts as a master regulator of the CWI pathway because it controls many downstream effectors that lead to changes in actin cytoskeleton

composition, proliferation, and cell wall biogenesis (Levin, 2005; Levin, 2011; Lipke and Ovalle, 1998). One of these primary effectors is protein kinase C, which activates the MAP Kinase (MAPK) cascade leading to the transcriptional output of the CWI pathway. Disruption of this cascade, specifically protein kinase C, leads to cell wall integrity and cell viability being compromised.

In *S. cerevisiae,* the *PKC1* gene encodes the single PKC homolog and the structure and activity of protein kinase C has been characterized (Schmitz et al., 2002; Reinoso-Martín et al., 2003). It has multiple conserved regions that act as binding sites, which leads it to have a variety of substrates with different downstream effects. A *S. cerevisiae PKC1* deletion is lethal, but viability can be achieved with osmotic support such as the addition of sorbitol. Because *P. pastoris* has a single homolog of the *PKC* gene, it is an attractive target to study its role on cell wall integrity and secretion in *P. pastoris.*

Yeast Proteomics and Mass Spectrometry

A rapidly growing area of biological research is proteomics, the comprehensive and quantitative study of proteins. This includes the study of proteins' physical structure and function, as well as changes in protein expression that are due to biological changes or disturbances (Anderson and Anderson, 1998). Proteomic studies aim to characterize genes and cellular functions at the protein level. The utilization of proteomics has led to research advances in protein-protein interactions, eukaryotic metabolic physiology, and the development of therapeutic drugs. Proteomic studies include the identification and characterization of a single protein, as well as complex populations of proteins from specific cells or tissues. These are referred to as "targeted proteomics" and "shotgun proteomics", respectively (Kolsrud et al., 2012). Currently, the method of choice for many proteomic studies is mass spectrometry (MS).

For the analysis of complex protein mixtures, MS is the primary method used. For targeted proteomics, mass spectrometry can be applied to study protein-protein interactions, primary amino acid sequencing, as well as post-translational modifications.

Although mass spectrometry can be utilized for many types of analytes, the focus of this study will be on the analysis of proteins. All analytes, even proteins, must first be ionized in the gas phase in order to be measured with a mass spectrometer. Whole proteins' large molecular weight makes MS analysis of intact proteins relatively difficult to detect. Therefore, an essential step is the degradation of the proteins to smaller peptides, typically via proteases of known function, to be used for mass spectrometry-based proteomics. For example, trypsin is a serine protease that mainly cleaves peptide chains at the carboxyl side (C-terminus) of lysine and arginine residues, which will produce peptides in a predictable manner. This protein digestion is a final step in the protein sample preparation procedure that precedes analysis on a mass spectrometer.

Typically, a mass spectrometer consists of three main compartments: an *ion source* that ionizes the analytes, a *mass analyzer* that detects the mass-to-charge ratio (*m/z*) of the ionized analytes, and a *detector* that detects the quantity of ions at each *m/z* ratio. For proteomic-based mass spectrometry, two common techniques are used to ionize peptides prior to analysis: electrospray ionization (ESI) is used for analyzing complex protein mixtures in solution (Fenn et al., 1989), while matrix-assisted laser desorption/ionization (MALDI) is used to sublimate a relatively simple protein mixture into the gas phase from a crystalline solid-phase matrix (Aebersold and Mann, 2003). Because proteins are in solution for ESI-MS, it is usually coupled with liquid chromatography for separation of the complex protein mixture based on ion-exchange, size of the protein/peptide, and/or partitioning during chromatographic separation

(Aebersold and Mann, 2003). The mass analyzer separates the ions according to their corresponding m/z ratio. The mass analyzer also has an influence on the mass accuracy of the ions, the resolution between two distinct *m/z* ratios, and sensitivity to the quantity of ions at each m/z ratio. There are different types of mass analyzers with different functions, and each has its own advantages and disadvantages over the others. Advanced mass spectrometers even combine two or more mass analyzers in order to have a more comprehensive analysis of the ionized peptides. ESI is usually coupled with ion trap analyzers that 'trap' the ionized peptides in a compartment for a specific time period, and then they are detected for subsequent MS or MS/MS analysis. MALDI is often coupled with time-of-flight (TOF, MALDI-TOF) analyzers that accelerate the ions, and measures the time it takes them to hit the detector. The time of flight is dependent on the mass-to-charge ratio, as well as their mass. Most importantly, it allows for the generation of information-rich mass spectra from the ionized peptides, as well as even further spectra of produced peptide fragments from the original ionized peptides (Kolsrud et al., 2012).

Subsequent fragmentation is performed on a prioritized selection of original ionized peptides that show a high intensity during detection. Tandem mass spectrometry (MS/MS or MS^2) is then performed in the mass spectrometer. When selected for MS/MS, the isolated peptide ion is fragmented via high-energy collisions with inert gas, and the fragments with specific *m/z* ratios produced are illustrated via a second mass spectra. This MS/MS data, which includes both MS and MS/MS spectra, are then used to compare protein sequence databases that can produce hypothetically digested proteins and their produced fragments. From this *in silico* comparison, the identity of the peptides - and the original proteins that they came from - can be determined. This output is a "protein hit list". Because the above-mentioned MS-based proteomic techniques are dependent on protein sequence databases, it limits these types of

proteomic research to species that have already been thoroughly sequenced with their data available online. Recently, an extensive 1.3-hour proteomic analysis of the entire *P. pastoris* proteome showcased the modern technological advancements of MS-based proteomic research with the utilization of an Oribtrap Tribrid mass spectrometer (Hebert et al., 2014). The MS analysis of the yeast proteome detected almost 4000 proteins in total.

As leading eukaryotic model organisms in biology, yeasts have been shown to be prime candidates for eukaryotic proteomic research; their attractive characteristics include being single-celled eukaryotes, as well as having been extensively studied and comprehensively sequenced, such as *S. cerevisiae* (Ghaemmaghami et al., 2003; Ho et al., 2002) and *P. pastoris* (De Schutter et al., 2009). *P. pastoris* is a host species for heterologous protein production that is no stranger to MS-based proteomic analysis. Due to recombinant protein expression occurring via secretion, the secreted proteome of *P. pastoris* is of central importance for characterization and optimization of recombinant protein expression. The *secretome* has been defined to be the proteome of the secreted proteins, including proteinaceous cellular machinery involved in secretion (Kim et al., 2007). The secretome of wild type and recombinant methanol-induced, fermentation cultures of *P. pastoris* has been previously characterized to illuminate the native secretome, as well as how it is affected by recombinant protein expression (Huang et al., 2011). Attempts to improve *P. pastoris* as a recombinant expression system have been made, such as the identification and deletion of a native contaminant protein that led to no significant differences in cellular growth and product formation (Heiss et al., 2013). The budding yeast species is certainly a considerable candidate for mass spectrometry-based proteomic research of eukaryotes.

Research Objectives and Aims

For it to continue to serve as a host system for industrial applications, optimization of heterologous secretion in *P. pastoris* must be held as a top priority. As of 2009, the yeast has been utilized to express thousands of recombinant proteins and 500 pharmaceutical proteins (Yang and Zhang, 2018). However, despite the success of the yeast as recombinant expression platform, the system is faced with limitations that arise partly due to strain and product-specific expression proteins (Yang and Zhang, 2018). Essentially, some recombinant proteins intended for secretion are instead either retained in the cell or degraded. Another challenge is that recombinant proteins expressed in *P. pastoris* may not have the same proper folding and post-translational modifications as the native protein; thus, this leads to reduced activity of the protein and even the potential for antigenic responses if the recombinant protein is intended for therapeutic application. Continued research is needed to overcome these impediments of optimized *P. pastoris* recombinant expression.

One avenue of exploration is the production of genetically modified strains that generate relatively increased expression of recombinant proteins. Recently, certain *P. pastoris* strains that displayed increased levels of secreted β-galactosidase reporter protein were developed through random genomic disruption mutations (Larsen et al., 2013). Of these mutant REMI (restriction enzyme mediated integration)-derived *beta-galactosidase supersecretion (bgs)* strains, one strain displayed elevated secretion of a variety of different reporter proteins. This strain, *bgs13,* had a random disruption of its *BGS13* gene that led to the supersecretion phenotype. It exhibited enhanced export of reporters such as horseradish peroxidase (HRP) with its α-helical-rich and metal-containing structure, as well as human secretory leukocyte inhibitor (SLPI) containing eight disulfide bonds.

The wild-type Bgs13p shares similarities with the protein kinase C of *S. cerevisiae*. The

predicted amino acid sequence shares 50% homology and 68% similarity to the Pkc1p (Naranjo et al., 2019). Because the Pkc1p is essential in the CWI pathway of *S. cerevisiae,* it initially raised the question if the variant's bgs13p (REMI-derived mutant protein) also influenced cell wall integrity. Colorimetric assays for released vacuolar alkaline phosphatase (ALP) confirmed a change in cell wall permeability, compared to the wild type cell. Treatment with the osmotic stabilizer sorbitol restored cell wall function by inhibiting ALP release (Larsen et al., 2013). Furthermore, the mutant *bgs13* strain displayed higher sensitivity to Congo red and Calcofluor white – compounds believed to interfere with cell wall assembly via binding to chitin – compared to the wild-type strain, an indication of cell wall defects (Naranjo et al., 2019). Transmission electron microscopy also identified the cell wall of the *bgs13* cells to be thicker compared to that of the wild-type parent cells. Therefore, it is possible that the mutant strain differs in cell wall structure and integrity due to a change in function of the variant bgs13p.

The *bgs13* allele may also play a role in the protein export and sorting process. A difference in export of a recombinant hybrid protein composed of an N-terminal maltose binding protein (MBP) and enhanced green fluorescent protein (EGFP) was seen in a past study (Moua et al., 2016). This hybrid fusion protein was proteolyzed prior to secretion in the wildtype strain, but secretion of MBP-EGFP was increased in the *bgs13* strain. In the wild-type background, intact MBP-EGFP was found to be localized in the vacuole via fluorescent microscopy. The mutant *bgs13* allele caused MBP-EGFP to be localized to parts of the secretory network prior to secretion, as expected. With fewer MBP-EGFP being sent to the vacuole, it is possible that improperly folded proteins are being secreted rather than being sent to the vacuole for degradation.

To give insight into the chromosomal *bgs13* mutation and its downstream effects, genetic

characterization of the chromosomal mutation was performed. Characterization and sequencing of the mutant *bgs13* mRNA indicated that it was a *Remi-bgs13* transcript, containing a 5' UTR, and 12 codons from the pREMI plasmid fused in-frame to codons 148 and after of the *BGS13* coding sequence (Naranjo et al., 2019). Thus, it encoded the resulting Remi-bgs13p that led to the supersecretion phenotype.

Attempts to generate a *BGS13* knockout strain were unsuccessful, suggesting that the *BGS13* gene is essential for viability (Naranjo et al., 2019). As such, further studies on the *bgs13* mutation cannot be performed in a knockout strain. Characterization of the mutant *Remi-bgs13* gene indicated that it is recessive to the wild type *BGS13* gene.

Because the Bgs13 peptide shares homology with *S. cerevisiae* Pkc1p, it was hypothesized to have protein kinase C activity. Compared to the wild-type Bgs13 peptide (with a C-terminal -myc-His6 fusion), cellular extracts containing the mutant Remi-bgs13 peptide (with a C-terminal -myc-His6 fusion) displayed lower protein kinase C activity compared to extracts containing the wild-type protein (Naranjo et al., 2019).

To test the hypothesis that decreased PKC activity led to increased secretion, a strain which overexpressed the wild-type Bgs13p-myc-His6 protein was transformed with a plasmid that expressed the SLPI reporter protein under the control of the *AOX1* promoter. Overexpression of Bgs13 peptide reduced secretion of SLPI approximately two-fold compared to a strain with wild-type levels of Bgs13 peptide (Naranjo et al., 2019). These experiments suggest that secretion is possibly regulated by protein kinase C activity of the Remi-bgs13 peptide. To determine if different cellular localization influenced secretion, C-terminal EGFP fusions of wild-type Bgs13 and Remi-bgs13 peptides were produced. Fluorescent microscopy observed that wild-type Bgs13p-EGFP was primarily localized at the neck and bud of dividing

cells, whereas Remi-bgs13-EGFP was localized at the periphery of the cell (Naranjo et al., 2019). As of the time of this writing, there is uncertainty whether the supersecretion phenotype of the *bgs13* strain is due to a change in protein kinase C activity or because of the difference in localization of the mutant protein.

Overall, it is not apparent whether the cell wall structure changes are an *effect* of the mutant bgs13 protein activity that is not related to increased protein secretion, or the *cause* of increased secretion. The long-term goal of this research is to uncover information regarding the role of the *BGS13* gene in secretion and cell wall integrity, and how the mutation in the *bgs13* strain leads to increased secretion of recombinant proteins.

Here, I aim to examine the role of the encoded Bgs13 protein in the secretion pathway by analyzing the mutant Remi-bgs13 protein's effects that lead to a cell wall integrity defect, increased secretion, and changes in the unfolded protein response. To address the cell wall defect displayed by the mutant strain, I compare the population of proteins found in the extracellular media, and those extracted from the cell wall via a dithiothreitol (DTT) extraction for both wild type and *bgs13* strains grown as shake flask cultures. I also examine the relative cell wall porosity to give further insight on any possible changes or differences in cell wall architecture of the two strains. Furthermore, due to the apparent differences observed in proteins found in the extracellular medium and in cell wall structure, changes in the UPR were also analyzed.

My results explicate, in part, the role of the modified Bgs13 protein and its activity in the CWI pathway, its effects on the cell wall structure, and proteins that were found in the extracellular medium. This research further illuminates how enhanced secretion can be achieved in a valuable yeast recombinant host system such as *P. pastoris*.

Chapter 2: Materials and Methods

Strain and Culture Conditions

All yeast strains used in this research, along with their corresponding genotypes, have been listed in Table 1. The *P. pastoris* strains yJC100 and yGS115 were derived from the original *P. pastoris* wild-type strain NRRLY11430 (Northern Regional Research Laboratories, US Department of Agriculture, Peoria, IL). Yeast strains were cultured in YPD (1% yeast extract, 2% peptone, 2% glucose), BMGY (buffered minimal medium with 0.5% methanol, 2% peptone, and 1% yeast extract), BMMY (buffered minimal medium with 0.5% methanol, 2% peptone, and 1% yeast extract), supplemented with the necessary amino acid(s) and 0.5% methanol. Antibiotic resistance in yeast was verified with either 100 µg/mL Zeocin or 0.5 mg/mL G418. Yeast strains were grown at 30°C unless otherwise stated. Yeast cultures were incubated in a New Brunswick Scientific C25 Incubating Shaker (Edison, NJ) at the indicated temperature with shaking at 225 RPM.

Table 1: *Pichia pastoris* Strains

Strain	Genotype
yDT39	*his4, met2,* ß-gal
ybgs13	*his4, met2,* Zeo^R, ß-gal
yJC100	Wild-type (haploid)

Mass Spectrometry and Proteomics of Proteins Found in the Extracellular Media: Protein Quantitation, Denaturation, Digestion, and Desalting

yDT39:pKanJV4 (wild-type,) and ybgs13:pKanJV4 cultures were grown overnight in YPD medium to stationary phase. Optical density measurements were taken, and 5.0 OD_{600} units of each culture were pelleted and resuspended in 10 mL of buffered minimal medium with dextrose (BMD) with histidine and methionine. Cultures were grown for 24 hours, then 50 OD_{600} units of wild-type culture and 100 OD_{600} units of *bgs13* culture (to compensate for lower growth rate) were pelleted, resuspended in 50 ml of buffered minimal medium with methanol (BMM) with histidine and methionine. Cultures were induced for 48 hours at 28°C with shaking (225 rpm), with methanol added to 0.5% 24 hours post-induction to compensate for losses from evaporation and metabolism. At harvest, the OD_{600} of each culture was measured, cells were centrifuged, and the supernatant was filtered (0.22 microns) to remove any remaining cell particulate. Cell pellets and supernatants were immediately frozen and stored at -80°C.

Supernatant total protein concentrations were measured at 280 nm for both strains using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Triplicate aliquots were made, containing 2 mg of protein and each replicate was spiked (1 part spiked reference proteins : 500 part protein) with protein standards including bovine serum albumin (BSA) (Pierce, USA) and horse myoglobin (Sigma Life Science, USA) for subsequent label-free proteomics quantitation analyses. Aliquots were precipitated using acetone (-20°C for 3 hours). Proteins were dried, pelleted, then resuspend in 200 µL 3M urea/100 mM Tris (pH7.8). Precipitated protein concentration was determined using the same methods above, then all samples were diluted to standardize concentrations prior to proteomics sample preparation (0.5µg/µL; 50µg total protein). Samples were reduced in 5mM dithiothreitol (DTT) (Gold Biotechnology, USA) for 30 minutes

at room temperature (RT) and alkylated using 15 mM iodoacetamide (IAA) (Sigma, USA) for 30 minutes in the dark at RT. Unreacted IAA was quenched by adding 20 µL of 200 mM DTT and incubating for an additional 30 minutes. Each reaction was then diluted with 3 volumes of sterile water (~750 µL) to reduce urea concentration to < 2 M, then digested overnight at 37 °C using trypsin (Promega, USA) (1 part trypsin : 10 parts protein). The digest was halted with the addition of trifluoroacetic acid (TFA) and digested peptides were purified using OMIX C18 spin columns according to the manufacturer's instructions (Agilent Technologies, USA). Samples were then diluted and lyophilized. Lyophilized samples were resuspended in 40 µL of 0.1% formic acid in HPLC grade water; peptide samples were diluted to 150 ng/µL. All samples were stored at −80 ◦C until mass spectrometry analysis.

HPLC-MS/MS

For each peptide sample, 5 µl were loop injected by a Dionex Ultimate 3000 autosampler onto a reversed-phase trap column (Acclaim™ PepMap™ 100 C18 LC column; 75 µm i.d. x 2 cm, 3 µm particle size, 100 A pore size, Thermo Fisher Scientific, USA). Peptides were bound and then eluted by using a reversed-phase analytical column (EASY-Spray™ C18 LC column; 75 µm i.d. × 15 cm, 100 A, Thermo Fisher Scientific, USA) held at 35°C for HPLC. Flow rates were kept at 300 nl/min with each sequencing run set to 140 minutes. Solvents A and B were 0.1% formic acid in water and in acetonitrile, respectively. Solvent B was used as following: 3% for 5 min, 3%–28% for 75 min, 28%–45% for 25 min, 45%–95% for 5 min, 95% for 5 min, return to 3% for 5 min, followed by 2% for 25 min.

Mass spectrometry analysis was performed using an Orbitrap Fusion™ Tribrid™ mass spectrometer equipped with an EASY-Spray™ ion source (Thermo Fisher Scientific, USA) operated in a data dependent acquisition (DDA) manner via Xcalibur 4.0 software (Thermo

Fisher Scientific, USA). Birefly, MS1 spectra were resolved by the Orbitrap; precursor ions selected via DDA were quadrupole-filtered and fragmented using HCD. Instrument and data acquisition settings were the same as previously reported (Khudyakov et al., 2018), with the exception of the scan range (200-1400 Da), MS1 Orbitrap resolution (120,000), scan time (10-130 minutes), MS2 Orbitrap resolution (30,000), and MS2 max injection time (150 ms).

Protein Identification

Analysis of tandem MS data was performed via Proteome Discoverer 2.2.0.388 (Thermo Scientific, USA). Peptide spectra were searched against the complete UniProt SwissProt database (downloaded on 2/13/2018) using SEQUEST. To identify any associated contaminants that may have arose during sample preparation, spectra were searched against the common Repository of Adventitious Proteins (cRAP, https://www.thegpm.org/crap/index.html). Search parameters were the same as previously reported (Khudyakov et al., 2018). False discovery rate (FDR) for peptide spectral matches (PSMs) and peptides were estimated by searching reversed decoy databases that were generated from the Uniprot SwissProt and cRAP databases. Results were filtered to remove identified contaminants. Peptides with target FDR < 1% were retained. For proteins to be categorized as "unique", they required 2 or more unique peptides that mapped back to the spectra.

Protein Quantitation

Protein quantitation was performed using Proteome Discoverer 2.2.0.388 (Thermo Scientific, USA). To align chromatographic runs for each biological condition, the feature mapper node was used with a maximum retention time shift of 10 minutes, a mass tolerance of 10 ppm, and a minimum S/N threshold of 5 for feature linking mapping. MS^2 data was filtered

to retain only proteins that had 2 or more unique peptide hits and were identified in all samples with high confidence. Biological replicates were grouped by condition. Quantitative abundances were calculated, normalized to reference proteins (BSA and horse myoglobin), and scaled with a label-free method using the Precursor Ion Quantifier node in Proteome Discoverer 2.2 (see methods). Abundance ratio calculations were made by using summed abundance values. An analysis of variance (ANOVA) on individual proteins was performed to determine whether protein abundances significantly ($p \leq 0.05$) differed between samples. Ratio abundances and $\log_2$fold-changes were calculated for the sample comparisons between wild-type and mutant strains.

DTT Extraction of Cell Wall Proteins

Dithiothreitol (DTT)-extracts of wild-type (yDT39:pKanJV4) and *bgs13* (ybgs13:pKanJV4) cells were prepared according to a previously described protocol (Scrimale et al., 2009). Briefly, 40 OD_{600} cells were grown to early stationary phase (OD_{600}~10) overnight in either BMM or BMMY, harvested by centrifugation (5 minutes, 300 ×*g*), washed twice with 10 mL water, and resuspended (0.5 OD_{600} equivalent/µl) in extraction buffer (50 mM Tris, pH 7.5, and 5 mM DTT). As negative controls, both strains were resuspended in 50 mM Tris pH 7.5 alone. The cell suspension was shaken in a multi-vortex apparatus for 2 h at 4°C. The supernatants were used for further analysis. An aliquot of the supernatant was removed, mixed with 2X Laemelli sample buffer, boiled for 5 min at 100°C, and fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) (BioRad mini-Protean TGX 4–20% gradient gel). Fractionated proteins were visualized by silver stain according to the manufacturer's protocol (Pierce Silver Stain kit; Thermo Scientific).

Mass Spectrometry and Proteomics of Proteins Extracted from the Cell Wall: Protein Quantitation, Denaturation, Digestion, and Desalting

From the DTT extraction of cell wall proteins, supernatant total protein concentrations were measured at 280nm for both strains using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Duplicate aliquots were made, containing 120 µg of protein. Aliquots were precipitated using acetone (-20°C for 3 hours). Proteins were dried, pelleted, then resuspended in 200 µL 3M urea/100 mM Tris (pH7.8). Precipitated protein concentration was determined using the same methods above, then all samples were diluted to standardize concentrations prior to proteomics sample preparation (approx. 0.21 µg/µL; 25 µg total protein). Prior to tryptic digest sample preparation, each replicate was spiked (1 part spiked reference proteins : 500 part protein) with protein standards including BSA and horse myoglobin for subsequent label-free proteomics quantitation analyses. Samples were reduced in 5 mM dithiothreitol (DTT) (Gold Biotechnology, USA) for 30 minutes at room temperature (RT) and alkylated using 15 mM iodoacetamide (IAA) (Sigma, USA) for 30 minutes in the dark at RT. Unreacted IAA was quenched by adding 20 µL of 200 mM DTT and incubating for an additional 30 minutes. Each reaction was then diluted with 3 volumes of sterile water (~750 µL) to reduce urea concentration to < 2 M, then digested overnight at 37 °C using trypsin (Promega, USA) (1:10, trypsin to protein). The digest was halted with the addition of trifluoroacetic acid (TFA) and digested peptides were purified using OMIX C18 spin columns according to the manufacturer's instructions (Agilent Technologies, USA). Samples were then diluted and lyophilized. Lyophilized samples were resuspended in 40 µL of 0.1% formic acid in HPLC grade water; peptide samples were diluted to 150 ng/µL. All samples were stored at −80 ◦C until mass spectrometry analysis. Subsequent HPLC-MS/MS, protein identification and protein

quantitation procedures and parameters were the same as those performed for the extracellular media.

Cell Wall Porosity as Determined by a Polycationic Assay

The permeability of the wild-type (yDT39:pAM1) and *bgs13* (ybgs13:pAM1) cell wall was measured using a protocol adapted for several different fungal species (Walker et al., 2018). The assay was performed on a mutant strain (FWK1) with a known cell wall defect in the *OCH1* gene, and its parent strain (Ku70) for comparison (Krainer et al, 2013). Briefly, overnight cultures of cells grown in YPD were diluted in fresh medium to 0.1 OD_{600} at 28°C and then allowed to grow to approximately 1 OD_{600}. For each strain, approximately 8 OD_{600} of cells were centrifuged @ 3500 *xg* for 7 min at room temperature. The supernatant was discarded, and the cell pellet was washed three times with 0.22 µm sterile-filtered milliQ water. The cells were then resuspended in 10mM Tris, pH 7.4 to a final OD_{600} of approximately 2.0 and the resuspension was divided into three 1.5 mL centrifuge tubes with an approximate volume of 0.9 mLs each. To each 0.9 mL cell suspension, the following were added, either: 1) 100 µL of 10x poly-L-lysine (Sigma-Aldrich, 30-70 kD, 100 ug/mL in 10mM Tris, pH 7.4) 2) 100 µL of 10x DEAE Dextran (Sigma-Aldrich, 500 kD, 50 ug/mL in 10mM Tris, pH 7.4) or 3) 100 µL of 10mM Tris, pH 7.4. The cell suspensions were then incubated at 28°C for 30 min at 200 RPM in shaking incubator. The cells were spun down for 2 min at 10,000 *xg* two times to isolate the supernatant. The A_{260} reading of each supernatant was taken to measure the UV absorbing compounds, using the supernatant from cells incubated in only 10 mM Tris buffer, pH 7.4 as a blank. The assay was performed on the bioreplicates samples in duplicate. The relative cell wall porosity was defined (%) = [(A_{260} of DEAE-dextran)/(A_{260} of poly-L-lysine)] × 100% (Tang et al., 2016).

Cytoplasmic and Membrane-Associated Protein Extraction from *P.* pastoris Strains yDT39:pAM1 and ybgs13:pAM1

Protein extractions from soluble cytoplasmic and insoluble membrane-associated fractions were performed according to a previous extraction procedure (Damasceno et al. 2009). Cells (OD_{600}=4) stored at -80°C were thawed, washed in phosphate-buffered saline (PBS) pH 7.4, and then resuspended in yeast breaking buffer [(50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, and 5% (v/v) glycerol]. An equal amount of acid-washed glass beads was added, and cells were lysed by vortexing at max speed ten times for 1 minute with 1-minute intervals on ice. The lysate was centrifuged at 10,000 *x*g for 30 minutes at 4°C. The supernatant, containing cytoplasmic proteins, was collected and designated as the cytoplasmic fraction. The remaining pellet with cell debris and glass beads was resuspended in 100 µL of breaking buffer containing 2% (w/v) SDS and centrifuged at 4000 *x*g for 5 minutes at 4°C. The supernatant was collected as designated as the membrane associated fraction. The extraction and detection of PDI was also performed on yJC100:pAM1:pPICZPDI as a positive control, a strain which overexpresses PDI (Li et al., 2010).

Polyacrylamide Electrophoresis and Western Analysis

Either equivalent amounts of protein or volumes of extracellular medium from equivalent numbers of cells were run on sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). The gels were either stained for total protein visualization using either the PageBlue (Thermo Scientific, Rockford, IL), Silver Snap II (Pierce, Rockford, IL) stains, or analyzed by western analysis.

For westerns, proteins were transferred onto nitrocellulose membrane using a an iBlot® apparatus (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Immunoblots were processed as follows. The membrane was soaked in 1X PBS while the SNAP ID 2.0 Protein Detection System blot holder (Millipore, Billerica, MA) was wet with water. The nitrocellulose membrane was placed on the blot holder, followed by a spacer sheet. The closed blot holder was placed onto the SNAP ID apparatus attached to a vacuum. With the vacuum turned on, 30 mL of I-Block (ThermoFisher Scientific, St. Louis, MO) solution (1xPBS, 0.2% I-Block, 0.1% Tween 20) was added to the membrane. The vacuum was turned off before incubating the membrane with 3 mL of I-Block solution containing the appropriate volume of primary antibody. After 10 minutes of incubation with the primary antibody, the vacuum was turned on again, and the membrane was washed three times with a total of 120 mL wash buffer (1X PBS, 0.1% Tween 20). The membrane was then incubated in 5 mL of I-Block containing the goat anti-mouse or goat anti-rabbit secondary antibody (Applied Biosystems, Foster City, CA) for 10 minutes with the vacuum off. Afterwards, the membrane was washed three times with wash buffer again, as previously described, before it was incubated for 5 minutes in a petri dish containing 20 mL of femtoLUCENT™ PLUS-AP 1X TBST (G-Biosciences, St. Louis, MO). A flat plastic-wrap surface was prepared where 2 mL of the femtoLUCENT™ PLUS-AP detection reagent was added dropwise onto the membrane. After 5 minutes of incubation at room temperature, the detection reagent was drained off. The membrane was placed into a plastic envelope or wrap and developed using the Bio-Rad ChemiDoc XRS+ Imaging System (Hercules, CA) with exposure times of 1-2 minutes. Signals were quantified with Bio-Rad ImageLab software.

Chapter 3: Results

ESI/MS/MS Analyses of Culture Media of 28°C Shake Flask-Grown Cultures

Initial research on the *bgs13* strain led to the observation of apparent differences in cell wall integrity and secretion, compared to the wild-type strain. A previous experiment had shown an increase in the levels of four out of five reporter proteins in the *bgs13* strain. Therefore, analyzing quantitative differences in the population of proteins found in the extracellular media (ECM) was the first approach in the characterization of the mutant *bgs13* strain. To identify and quantitate the relative abundance of proteins present in 28°C shake flask culture medium of both mutant *bgs13* and DT39 (wild-type; WT) cultures, samples of each strain's post-induction ECM were collected, solubilized in 3M urea, and subjected to an overnight digest with trypsin. Both strains were engineered to recombinantly express an MBP-EGFP fusion reporter protein, and its export was studied previously (Moua et al., 2016). Label-free quantitative mass spectrometry was performed by spiking the extracellular media from equal number of cells with a known amount of protein standards, bovine serum albumin (BSA) and horse myoglobin (1-part spiked reference proteins : 500-part protein). The produced peptides were then separated via nano-liquid chromatography, eluted and ionized by ESI for MS/MS analysis. The MS fragmentation spectra were searched against the entire Uniprot Swissprot database. Between wild-type and *bgs13* protein samples, relative protein abundance was calculated via ProteomeDiscoverer 2.2 by detecting and relating peptide signals at the MS^1 level. Within both wild-type and mutant *bgs13* extracellular media, a total of 335 proteins were identified with high confidence (after filtering of the contaminants identified via the cRAP database), with 2+ unique peptides mapping back to each identified protein, across all six technical replicates (Table 2, Figure 1). Eleven proteins were identified to be either uncharacterized or hypothetical proteins after BlastP analyses.

Table 2: Summary statistics of proteins found in the extracellular media (ECM)

Proteins identified	949
Proteins with >2 unique peptides	335
Protein groups	613
Peptide spectral matches (PSMs)	36,268
MS/MS spectra	106,346

Since the *bgs13* strain is hypothesized to be a possible supersecreter strain, the relative abundance of the 335 identified proteins found in the post-induction extracellular media of both strains was determined. Differential abundance of an identified protein was calculated as the ratio of the protein's grouped (i.e. average) abundance in the *bgs13* ECM to the protein's grouped abundance in the wild-type ECM. A fold change in differential abundance of proteins found was calculated by taking the log2 of the abundance ratio. The majority of identified proteins were found to be higher in abundance in the *bgs13* strain (Figure 1).

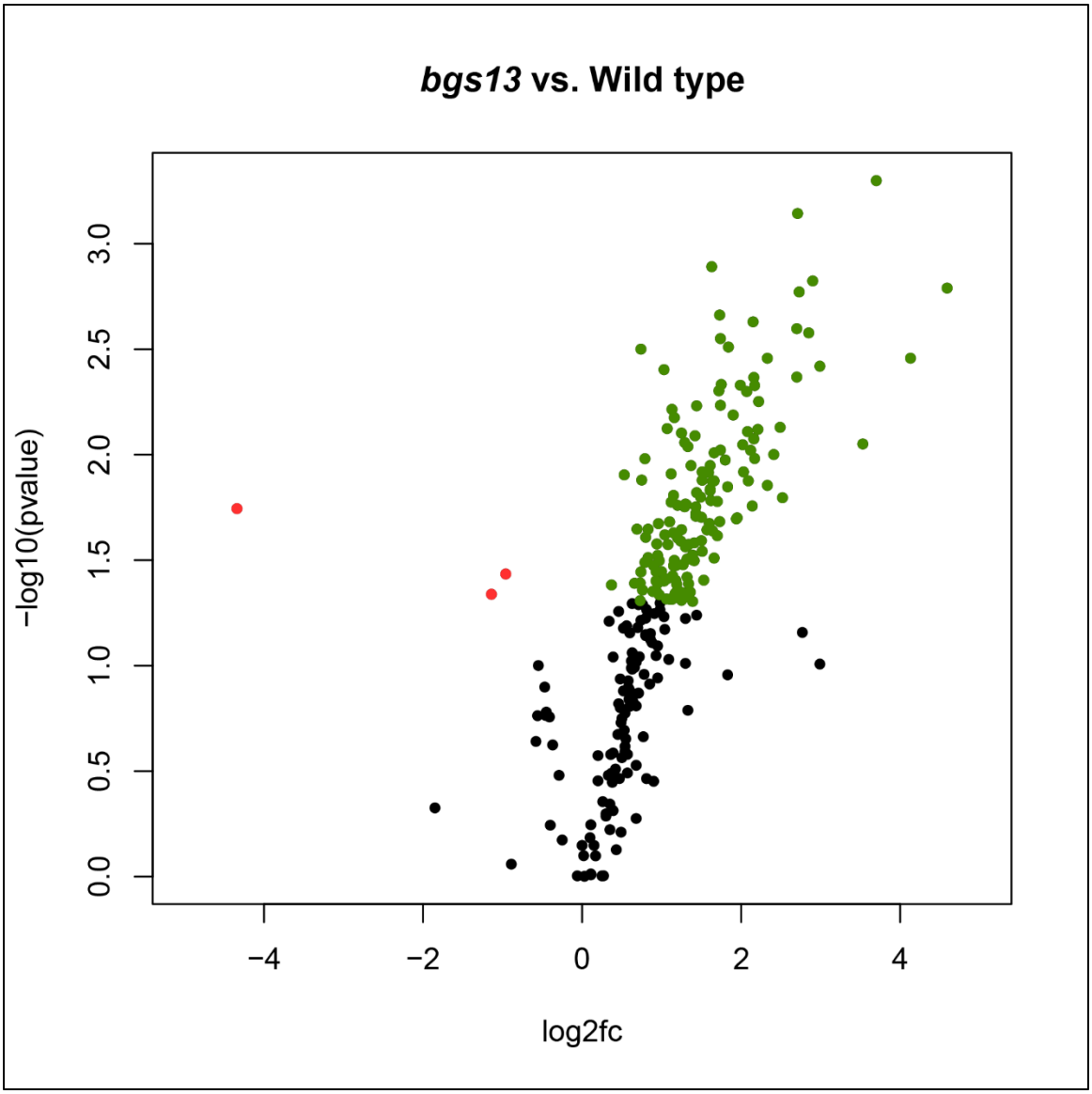

Figure 1: Differential protein populations found in the extracellular media of shake flask cultures grown at 28°C. A volcano plot illustrates the differential abundance of proteins found in the extracellular media post-methanol induction. Differentially abundant proteins of statistical significance ($p \leq 0.05$) are shown in red (lower abundance in *bgs13*) and green (higher abundance in *bgs13*). X-axis, log2(fold-change): fold-change in abundance between the two populations, wild-type and mutant bgs13. Y-axis, -log10(p-value): The statistical significance in the fold-change of abundance. Fold changes are calculated by taking the log2 of the abundance ratio (*bgs13*:wild-type); for each strains' three technical replicates, the abundance of each identified protein was grouped (averaged) for use in the abundance ratio and fold-change.

Of the recombinant hybrid reporter protein MBP-EGFP, enhanced green fluorescent protein (EGFP) and maltose binding protein (MBP) were found to have the highest abundance ratios with 24.043 and 17.524, respectively. Through manual annotation of the protein hits provided from Proteome Discoverer 2.2, nine proteins that were previously characterized as secreted or contain a signal peptide sequence were identified (Table 3). These were found in a past proteomic study on the secretome of methanol-induced *P. pastoris* cultures (Huang et al. 2011). These proteins include cell wall-associated proteins such as 1,3-ß-glucanosyltransferase (ATY40_BA7501626) and exo-ß-1,3-glucanase (*EXG*), as well as other native secreted proteins such as vacuolar proteases (BA75_01312T0, *PRB1* and BA75_03408T0, *PEP4*) and a known host-cell contaminant (BA75_03408T0, *PRY2*). Of the 335 proteins that were identified in both strains' extracellular media, many are of intracellular origin. Differentially abundant intracellular proteins, those found at higher levels in *bgs13* ECM, include those associated with the UPR (Kar2p, *KAR2;* Protein disulfide isomerase, *PDI*) and protein folding and aggregation (BA75_00603T0, *CPR6;* BA75_00961T0, *SGT2;* BA75_01760T0, *HSP104;* BA75_02652T0, *STI1;* BA75_00236T0, ATY40_BA7500236). Furthermore, the *bgs13* ECM had alcohol oxidase (BA75_04486T0, ATY40_BA7504486, abundance ratio = 2.041; BA75_03165T0, ATY40_BA7503165, abundance ratio = 1.461) in higher abundance than in the wild-type ECM. The observed differential abundance of the identified proteins between the two strains indicates that there are underlying factors at work within the cell that lead to differences in the protein populations found in the extracellular media after induction of heterologous expression.

Table 3: Summary information of identified representative proteins in the extracellular media that contain a signal peptide (SP) sequence.
**Komagataella pastoris (K. pastoris)* is the reassigned name to *Pichia pastoris* and is used by the Unirprot Swissprot database.

Uniprot entry code (Accession)	Protein name	Gene name	Description	Abundance ratio (bgs13/WT)
A0A1B2J7P6	1,3-ß-glucanosyltransferase	ATY40_BA7501626 *(K. pastoris*)*	Involved in cell wall biosynthesis and morphogenesis	4.268
A0A1B2JAX4	BA75_02022T0	ATY40_BA7502022 *(K. pastoris)*	Cell wall protein that contains a putative GPI-attachment site	2.840
A0A1B2JF94	BA75_03408T0	*PEP4 (K. pastoris)*	Vacuolar aspartyl protease (proteinase A)	1.472
Q2TCV2	Exo-ß-1,3-glucanase	*EXG (K. pastoris)*	Involved in cell wall beta-glucan assembly and maintenance	1.306
A0A1B2J758	BA75_01312T0	*PRB1 (K. pastoris)*	Vacuolar proteinase B (YscB), a serine protease of the subtilisin family	1.075
A0A1B2J5U4	BA75_00070T0	*PRY2 (K. pastoris)*	Sterol binding protein involved in the export of acetylated sterols	1.014
A0A1B2J755	BA75_01624T0	ATY40_BA7501624 *(K. pastoris)*	Cell wall protein with similarity to glucanases	0.728
A0A1B2JDU3	BA75_03273T0	ATY40_BA7503273 *(K. pastoris)*	Mitochondrial outer membrane and cell wall localized SUN family member	0.724
A0A1B2JDF4	BA75_02994T0	*YDR262W (K. pastoris)*	Putative protein of unknown function	0.667`

Extraction and Analysis of Cell Wall Proteins

Silver stain of DTT-extracted cell wall proteins. Because the *bgs13* strain showed compromised cell wall integrity in previous work (Larsen et al., 2013), differences in the cell wall structure may reveal more information on possible factors that could be leading to the differential abundance of proteins in the extracellular media. To characterize the structural differences in the cell wall between the wild-type and mutant strains, an analysis of extractable proteins from the cell wall was utilized. Dithiothreitol (DTT) was used as the extraction agent due to its ability to reduce and release dithiol-linked proteins of the cell wall. It is expected that defects in the cell wall composition or structure would lead to an increase in DTT-extractable proteins (Scrimale et al., 2009). To perform the DTT extraction, wild-type (yDT39:pKanJV4) and *bgs13* (ybgs13:pKanJV4) cells, which both expressed MBP-EGFP, were first separated from the extracellular media via centrifugation, washed with sterile-filtered water, and incubated in an extraction buffer containing 50mM Tris, pH 7.5 and 5mM DTT. The supernatant fractions were analyzed by SDS PAGE.

An initial silver stain of fractionated DTT-extracted proteins derived from an equal number of wild-type and *bgs13* cells displayed a visibly increased amount of extractable proteins from the mutant cells (Figure 2). Novel protein bands seemed to be present from the *bgs13* extracts, and other protein bands appeared to increase in concentration from wild-type to *bgs13* such as those at 40-45 kDa. Cells grown in BMM media were compared with those grown in BMMY media containing yeast extract, with no observable outstanding differences. The increased amount of proteins liberated from the *bgs13* cells gave reason to analyze the DTT-extracted cell wall proteins as the next protein population for mass spectrometry analysis.

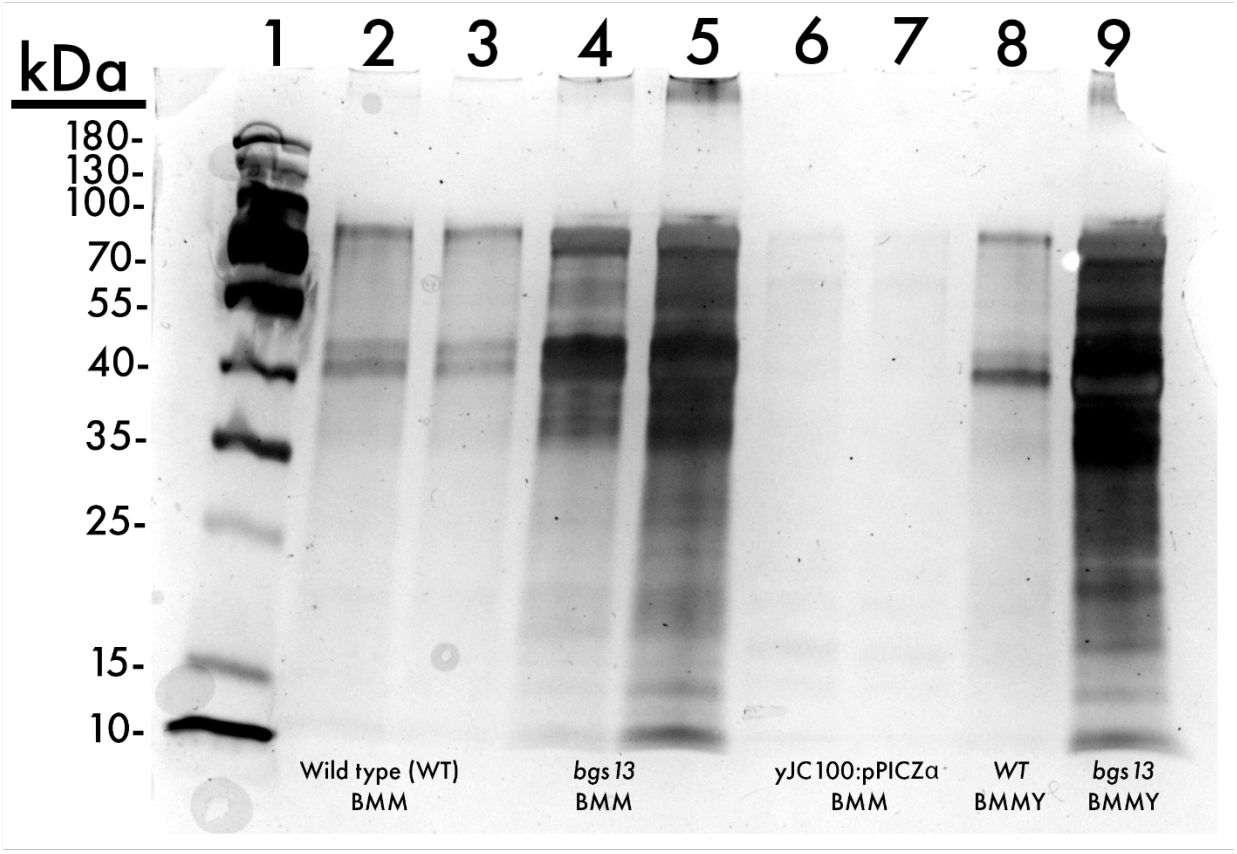

Figure 2: SDS-PAGE analysis of the proteins extracted from the cell wall, post-induction. After fractionation via SDS-PAGE, a silver stain was performed on proteins that were dithiothreitol (DTT)-extracted from the cell wall after removal of the extracellular media. Lanes: (1) molecular ladder; (2 and 3) wild-type, BMM media; (4 and 5) *bgs13* mutant, BMM media; (6 and 7): negative control strain harboring an empty vector, yJC100:pPICZα, BMM media; (8): wild-type, BMMY media; (9): mutant *bgs13*, BMMY media

ESI/MS/MS of DTT-extracted cell wall proteins. The protein population extracted from the cell walls of both wild-type and mutant *bgs13* underwent sample preparation, tryptic digest and subsequent data analysis as was performed on the extracellular media. A total of 359 proteins were identified in the extracts of both strains with high confidence (after filtering of the contaminants identified via the cRAP database), with >2 unique peptides mapping back to each

identified protein, across all four technical replicates (Table 4, Figure 3). 23 proteins were identified to be either uncharacterized or hypothetical proteins after BlastP analyses.

Table 4: Summary statistics of proteins found from the DTT-extraction of the cell wall

Proteins identified	1,148
Proteins with >2 unique peptides	359
Protein groups	747
Peptide spectral matches (PSMs)	26,247
MS/MS spectra	79,448

In order to characterize any observable differences in cell wall integrity or composition that emerged due to the Remi-bgs13 hybrid protein, I aimed to quantitate the relative abundance of the 359 proteins extracted from the cell wall in both strains, *bgs13* and wild-type. The differential abundance of the 359 identified cell wall-extracted proteins was obtained, and fold changes in the differential abundance are graphically represented on a volcano plot (Figure 3).

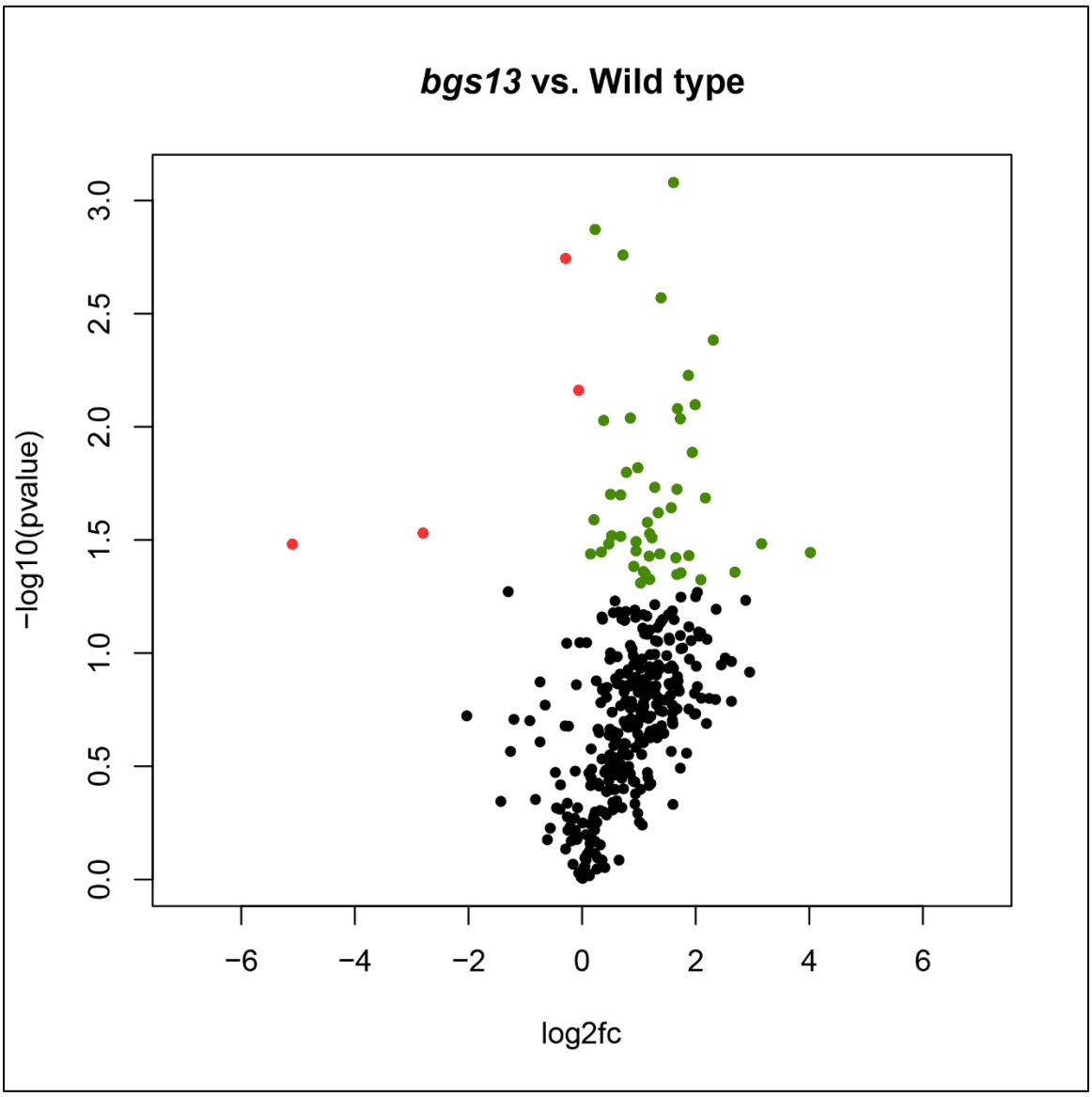

Figure 3: Differential protein populations found from the DTT-extraction of the cell wall from cells grown in shake flask cultures at 28°C. A volcano plot illustrates the differential abundance of proteins extracted from the cell wall post-methanol induction. Differentially abundant proteins of statistical significance ($p \leq 0.05$) are shown in red (lower abundance in *bgs13*) and green (higher abundance in *bgs13*). X-axis, log2(fold-change): fold-change in abundance between the two populations, wild-type and mutant bgs13. Y-axis, -log10(p-value): The statistical significance in the fold-change of abundance. Fold changes are calculated by taking the log2 of the abundance ratio (*bgs13*:wild-type); for each strains' three technical replicates, the abundance of each identified protein was grouped (averaged) for use in the abundance ratio and fold-change.

Similar to those found in the extracellular media, the majority of the 359 proteins were identified to be more abundant in the mutant *bgs13* cells relative to the wild-type extraction. The two parts of the hybrid recombinant reporter protein, EGFP and MBP, were found to be higher in abundance in the *bgs13* strain (abundance ratios of 2.262 and 1.977, respectively), but they were not the proteins with the highest abundance ratios. Through manual annotation of the protein hits provided from Proteome Discoverer 2.2, eight previously characterized cell-wall associated proteins were identified (Table 5). These include multiple isoforms of 1,3-ß-glucanosyltransferase with varying abundance ratios ranging from 3.710 to 0.418 (ATY40_BA7501626, *GAS1*, *GAS3*). Two of the three 1,3-ß-glucanosyltransferase isoforms encoded by ATY40_BA7501626 and *GAS1* displayed higher abundance in the *bgs13* extract. Other proteins that are involved with cell wall assembly and/or maintenance include endo-ß-1,3-glucanase (BA75_03970T0, *BGL2*), chitin transglycosylase (BA75_04772T0, ATY40_BA7504772), a cell-wall associated aspartic protease (BA75_00420T0, *YPS7*) and an uncharacterized glucanase-like protein (BA75_01624T0, ATY40_BA7501624) (Table 5). Many of the 359 proteins that were identified in both strains' cell wall extraction were of intracellular origin, such as key proteins involved in the UPR and protein folding including protein disulfide-isomerase (*PDI,* abundance ratio = 1.70) and Kar2p (*KAR2,* abundance ratio = 2.292), which were found to be more abundant in the *bgs13* extraction. Furthermore, proteases with SP sequences (BA75_01312T0, *PRB1*; BA75_03727T0, *LAP4*; BA75_03408T0, *PEP4*; BA75_05092T0, ATY40_BA7505092) and one with chaperone activity (BA75_02264T0, *NMA111*) were identified in the extraction from both strains. Alcohol oxidase (BA75_03165T0, ATY40_BA7503165, abundance ratio = 4.510; BA75_04486T0, ATY40_BA7504486, abundance ratio = 1.877) was higher in abundance in the *bgs13* mutant. Due to the apparent

differences in protein abundance obtained from the mass spectrometry analysis, I aimed to further characterize the cell wall by investigating any observable difference in cell wall porosity in relation to the Remi-bgs13 hybrid protein.

Table 5: Summary information of identified representative cell-wall associated proteins from the DTT-extraction of the cell wall. **Komagataella pastoris (K. pastoris)* is the reassigned name to *Pichia pastoris* and is used by the Unirprot Swissprot database.

Uniprot entry code (Accession)	**Protein name**	**Gene name**	**Description**	**Abundance ratio (bgs13/WT)**
A0A1B2JAX4	BA75_02022T0	ATY40_BA7502022 *(K. pastoris*)*	Cell wall protein that contains a putative GPI-attachment site	4.073
A0A1B2J7P6	1,3-ß-glucanosyltransferase	ATY40_BA7501626 *(K. pastoris)*	Involved in cell wall biosynthesis and morphogenesis	3.710
A0A1B2J5A4	1,3-ß-glucanosyltransferase	*GAS1 (K. pastoris)*	Involved in cell wall biosynthesis and morphogenesis	1.629
A0A1B2J7D2	BA75_00420T0	*YPS7 (K. pastoris)*	Putative GPI-anchored aspartic protease; involved in cell wall growth and maintenance	0.940
A0A1B2JJE0	BA75_04772T0	ATY40_BA7504772 *(K. pastoris)*	Chitin transglycosylase	0.721
A0A1B2J755	BA75_01624T0	ATY40_BA7501624 *(K. pastoris*	Cell wall protein with similarity to glucanases	0.657
A0A1B2JGR3	1,3-ß-glucanosyltransferase	*GAS3 (K. pastoris)*	Involved in cell wall biosynthesis and morphogenesis	0.418
A0A1B2JF97	BA75_03970T0	*BGL2 (K. pastoris)*	Endo-ß-1,3-glucanase; involved in incorporation of newly synthesized mannoprotein molecules into the cell wall	0.406

Cell Wall Porosity

We hypothesized that enhanced secretion may have resulted from an increase in cell wall porosity due to the structural changes detected by the DTT assay. In order to observe differences in strains engineered for recombinant protein expression, strains that expressed SLPI-myc-6xHis under the *AOX1* promoter, yDT39:pAM1 and ybgs13:pAM1, were chosen for the assay. Measurements of the cell wall porosity of the two *P. pastoris* strains, utilized two polycationic polymers with differently sized hydrodynamic radii. DEAE-dextran and poly-L-lysine both enhance the permeability of the cell membrane, thus leading to release of cytosolic UV-absorbing compounds such as nucleic acids. Poly-L-lysine (30-70 kDa, molecular mass) has a relatively small hydrodynamic radius and causes cell leakage independent of cell wall porosity. DEAE-dextran (500 kDa, molecular mass) causes cell leakage but its larger hydrodynamic radius limits its passage through the cell wall, making its effectiveness dependent on the cell wall porosity. The relative cell wall porosity was defined (%) = [(A_{260} of DEAE-dextran)/(A_{260} of poly-L-lysine)] × 100%. Therefore, a difference in cell wall porosity via this assay based on measurement of cell lysis was determined by the ratio between DEAE-dextran- and poly-L-lysine-induced release of UV-absorbing compounds measured at 260nm. For all strains, equal amounts of cells were separated from the extracellular media supernatant, washed, and then incubated in buffer containing either 1x DEAE Dextran or 1x poly-L-lysine. The A_{260} of the supernatant was measured for the relative porosity calculations. In addition to the yDT39 and *bgs13* strains, the polycationic porosity assay was also performed on a strain with a known cell wall defect, FWK1, along with its parent strain as controls. The FWK1 strain contains a deletion of the *OCH1* gene that encodes a mannosyltransferase. The results on these control strains validated the cell wall porosity (Table 6): compared to its parent, the FWK1 strain with the cell

wall defect displayed a ten-fold increase in relative porosity. Contrary to our expectations, the *bgs13* cell walls were found to be about 9.5% less porous than that of the wild-type cells (Table 6).

Table 6. Relative cell wall porosity as determined by a polycationic assay. A strain (FWK1) with a known cell wall defect caused by a deletion of the *OCH1* gene, and its parent strain (Ku70) were also analyzed for comparison. Data reflects the averages of duplicates.

Strain	Relative cell wall porosity %
yDT39:pAM1	31.9 ± 3.11%
ybgs13:pAM1	22.4 ± 2.92%
Ku70	12.2 ± 4.94%
FWK1	122 ± 14.0%

Changes in the UPR: Intracellular Protein Disulfide Isomerase (PDI) Expression

The apparent differences in relative protein abundance detected in the extracellular media and cell wall extracts, as well as the difference in cell wall porosity, can potentially be related to changes in the unfolded protein response. Furthermore, the presence of Kar2/Bip and protein disulfide isomerase protein (Pdip) in the ECM and cell wall suggests that the Remi-bgs13 protein leads to inefficient retention of these proteins in the ER, or that the release of these chaperones into these two locations is due to cell lysis caused by an altered cell wall. Because Pdip was found in higher abundance in the *bgs13* ECM and cell wall, it raised two possible scenarios: 1) If the mutant Remi-bgs13 protein led to increased Pdip expression, and sent Pdip to the cell wall and ECM due to inefficient retrieval, then the level of intracellular Pdip would be comparatively

similar inside the wild-type and *bgs13* cells, or 2) If Pdip was overexpressed, accumulated and retained in the ER and its release only occurred after cell lysis, then the intracellular levels of Pdip would be greater in the *bgs13* extraction. To investigate this aspect of the UPR, potential differences in the expression levels of the downstream UPR target, protein disulfide isomerase, were examined between wild-type and *bgs13* cells after induction of recombinant protein expression.

As a positive control, a strain overexpressing PDI from the *AOX1* promoter (yJC100:pAM1:pPICZPDI) was included in our study. Equal number of cells from yDT39:pAM1, y*bgs13*:pAM1, and the control were subject to protein extraction. Cells were lysed with glass beads and after centrifugation, the supernatant was collected as the "soluble" fraction. The remaining pellet was then extracted with breaking buffer and the resulting proteins were designated as the "insoluble membrane-associated fraction". Approximately equal amounts of protein from each strain's soluble and insoluble fractions were subjected to western blot analysis detection with a primary antibody against PDI protein (Pdip).

While the soluble fractions did not have significant amounts of Pdip, the insoluble membrane-associated fractions contained detectable levels of PDI (Figure 4). This is likely due to PDI being enclosed within the ER. The positive control from yJC100:pAM1:pPICZPDI, produced the strongest intensity. Pdip had a higher detectable intensity in the wild-type strain than in *bgs13* (Figure 4). However wild-type and *bgs13* Pdip levels were considered to be comparable, suggesting that similar amounts of ER-residential protein disulfide isomerase are present in both the wild-type and *bgs13* cells. Based on these results, this portion of the UPR was not upregulated in the *bgs13* strain.

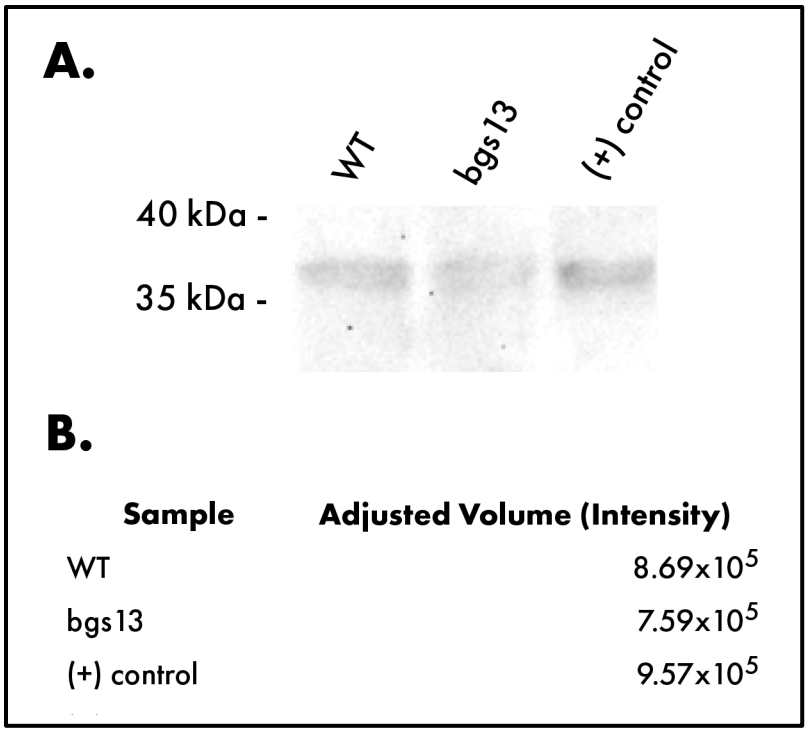

Figure 4: Intracellular protein disulfide isomerase (PDI) expression levels in *P. pastoris* strains after methanol-induced recombinant protein expression. A) Immunoblot of intracellular PDI levels extracted from insoluble membrane-associated fractions. Strains from left to right, "WT" (wild-type): yDT39:pAM1, "bgs13": ybgs13:pAM1, "(+) control": yJC100:pAM1:pPICZPDI. Exposure time: 5 minutes. B) Quantitative intensity of each strain's detected PDI protein band. Adjusted volumes were calculated using a global background subtraction of a single background volume (global volume subtraction calculates a single background intensity for the entire membrane). Area of detection (mm^2): 19.6181, number of pixels: 468, mean background (intensity): 1442.57.

Chapter 4: Discussion

Study Objectives

Pichia pastoris has been established as a reputable host for recombinant protein expression. As a single-celled eukaryote with the capabilities of achieving scalable growth and expression as well as programmed export of a recombinant gene product via secretion, it is at the forefront of expression systems used for biopharmaceutical production (Looser et al., 2015). Nevertheless, *P. pastoris* has the potential to be further optimized in order to heterologously express a wider variety of proteins, which can be attained by a deeper understanding of the yeast's mechanisms of secretion. Unfortunately, the most prominent limitation to this expression system is the occurrence of some recombinant proteins intended for secretion being either retained or degraded in the cell; this results partly from the proteins not achieving their intended native folding or correct post-translational modifications. One way of addressing this issue is to engineer genetically modified strains that can generate increased expression of recombinant proteins. The mutant *bgs13* strain, produced from a genomic disruption mutation (Larsen et al., 2013), displayed increased secretion of multiple recombinant reporter proteins – an indication that it may be a possible universal supersecreter strain. From the mutation in the *BGS13* gene that gave rise to this strain, the encoded Remi-bgs13 protein is a primary target to examine the underlying mechanisms that are involved in secretion. Illuminating the effects of the mutant Remi-bgs13 protein on enhanced secretion, cell wall integrity and changes to the UPR will provide insight into the role of the encoded Bgs13 protein in secretion.

Proteins Found in the Extracellular Media

The characterization of the secretion differences between the wild-type and *bgs13* strains began with a quantitative analysis of proteins found in the extracellular media after methanol-induced recombinant protein expression. The identification and characterization of proteins in the extracellular media was performed using a label-free quantitative mass spectrometry approach. In this regard, the relative quantity of the proteins present only in both strains were obtained. From the post-induction culture supernatant samples grown at 28°C, 335 proteins were identified in both the extracellular media of wild-type and *bgs13* strains (Table 2; Supplemental Table 1). This high number of proteins is composed mostly of those with an expected intracellular subcellular location and/or origin, which is possibly due to cell lysis that occurs during the process of growth in the shake-flask cultures. A previous proteomic analysis of fermenter grown, methanol-induced cultures also identified proteins of intracellular origin in the extracellular media (Huang et al., 2011). However, they did not identify as many as found here. Therefore, the higher number of intracellular proteins found in the extracellular media of our shake-flask cultures may be due to greater sensitivity that comes with improved instrument technology or the lack of control of growth conditions that bioreactors maintain. For shake-flask cultures, temperature and the agitation of the incubator are the main parameters that can be controlled; bioreactors can regulate wide range of growth conditions, such as the feeding of growth media, monitoring of methanol levels, oxygen levels, and pH monitoring (Cregg, 1999). A lack of constant, fixed growth conditions likely leads to a higher occurrence of cell lysis that causes a variety of intracellular proteins to be released into the extracellular media.

As expected, the hybrid reporter protein, MBP-EGFP, were found to be the most differentially abundant proteins between *bgs13* and wild-type strains, a result confirmed by western analysis (data not shown). Alcohol oxidase, a resident enzyme of peroxisomes, was

identified in the ECM of both strains because it is induced to an extremely high level when *P. pastoris* is grown on methanol as the sole carbon source (Lin-Cereghino and Cregg 2000). However, alcohol oxidase was found to be twice as abundant in the *bgs13* as the wild-type ECM. Furthermore, the majority of the 335 identified proteins were found to have an abundance ratio (*bgs13*/wt) of 1.0 or higher. As a reminder, the differential abundance of a protein was calculated as the ratio of the protein's average abundance in the *bgs13* ECM to the protein's average abundance in the wild-type ECM. Many secreted proteins, identified in previous publications (Huang et al., 2011; Mattanovich et al., 2009) were identified in this study (Table 2). Cell-wall associated proteins 1,3-ß-glucanosyltransferase (ATY40_BA7501626, abundance ratio = 4.268) and BA75_02022T0 (ATY40_BA7502022, abundance ratio = 2.840) were found to be considerably differentially abundant. This may be related to the role of the Bgs13 protein in cell wall integrity.

Vacuolar proteases (*PEP4, PRB1*) were also identified to be more abundant in the *bgs13* strain. The increased release of these proteases by the mutant raises concern for the expression and purification of recombinant protein expression; higher protease levels in the extracellular medium may lead to secreted proteins being degraded. Therefore, to further optimize the *bgs13* strain for recombinant protein expression via secretion, the implementation of protease-deficiency should be explored (Cereghino and Cregg, 2000; Idiris et al., 2006).

Interestingly, the endogenous protein secreted at highest levels by wild-type *P. pastoris,* a protein (Heiss et al., 2013) encoded by *PRY2,* was found to not be differentially secreted, with an abundance ratio of 1.017. This peptide is considered a contaminant when trying to purify a recombinant protein. Despite the *PRY2* gene product being a native secreted protein that can be induced by different stress conditions and upregulated by the UPR (Heiss et al., 2013), the

mutation in the *BGS13* gene did not have a considerable effect on its export into the extracellular media. This proves to be a good indication that the *bgs13* strain does not increase the export of all proteins intended for secretion. Not enhancing the secretion of a native contaminant peptide certainly is an attractive aspect of the *bgs13* secretion strain.

Although a high number of intracellular proteins were identified in the media, many were of interest. For example, UPR-associated proteins such as Kar2/Bip and protein disulfide isomerase (Pdip) were found to be higher in abundance in the *bgs13* ECM. In a *P. pastoris* strain that overexpressed the UPR-inducing *HAC1* gene, leading to an increase in the UPR, both Pdip and Kar2/Bip were identified in the culture supernatant (Guerfal et al., 2010). Another study also found that full-length and truncated Kar2/Bip and Pdip in the extracellular media after recombinant expression of an insulin precursor (Roth et al., 2018). One explanation is that these proteins, which are intended to remain in the ER, might likely be sent out of the ER due to excessive ER stress caused by recombinant protein occupancy expression (Liu et al., 2005; Guerfal et al., 2010). A second possibility is that Kar2/Bip could be bound to the aberrant proteins as they traverse through the secretory pathway (Roth et al., 2018). Within the *bgs13* cells, higher amounts of Pdip could possibly be packaged into secretory vesicles during the secretion process and being sent out into the media along with other proteins intended for secretion (Huang et al., 2011). In addition to the UPR-associated proteins, many heat shock response chaperone proteins involved in misfolding/aggregation were identified. Detection of heat shock proteins and chaperones in higher abundance in the *bgs13* ECM, such as STI1p and Hsp42p, suggests the increased presence of misfolded proteins being produced in the cells. It is important to note that the *identification* of these proteins occurred in both strains, but their *relative abundance* differs.

Overall, the *bgs13* strain released the majority of the identified proteins in higher abundance than the strain with the wild-type Bgs13 protein. This included EGFP and MBP reporter proteins, known secreted proteins, cell-wall associated proteins, UPR-associated proteins, and even an array of heat shock proteins. To address whether these differences in the protein populations of the post-induction ECM were due to a change in cell wall integrity that leads to a release of more protein, we next aimed to perform a deeper analysis of the *P. pastoris* cell wall of these two strains and its relationship to the ECM protein population.

Cell Wall Protein Extraction: Silver Stain Results

Initial observations of cell wall integrity being compromised in the *bgs13* strain (Larsen et al., 2013) led to the hypothesis that the pREMI-derived mutation in the *BGS13* gene caused a change in the cell wall architecture, leading to the supersecretion phenotype. In addition, transmission electron microscopy (TEM) of the *bgs13* cells revealed a thicker cell wall compared to the wild-type cells (Naranjo et al., 2019). Thus, further analysis of the cell wall and its structure were needed. DTT-extracted cell-wall associated proteins from both the wild-type and *bgs13* cells were compared and analyzed by SDS-PAGE to provide insight into any structural changes of the cell wall that would lead to liberation of proteins. In order to extract more details about these differences, label-free mass spectrometry analysis was performed on these protein populations.

Cell Wall Protein Extraction: MS/MS Analysis

From the cell walls of cells separated from the extracellular supernatant after methanol-induced recombinant expression, label-free quantitative mass spectrometry identified 358 proteins present in both the *bgs13* and wild-type extractions Like the protein populations of the

extracellular media, most of the proteins were found to be more abundant in the *bgs13* cells. This could be related to the thicker cell walls of the *bgs13* cells, which leads to higher retention of proteins within the structure. Again, this number is mainly comprised of proteins with an intracellular subcellular location and/or origin. Unlike the ECM protein population, this may not be due to a cell lysis-related cause because the cells were washed prior to the DTT extraction. However, cells may have lysed during the DTT extraction process. Proteins with an intracellular origin that were found to be extracted from the cell wall include the UPR-associated Kar2/Bip and Pdip. Both were found in higher abundance in the *bgs13* cell wall with abundance ratios of 2.292 and 1.700, respectively. Their identification here is most likely related to their presence in the media since they must pass through the cell wall prior to secretion out of the cell. Alcohol oxidase was another intracellular protein that was extracted from both strains and had a higher abundance in the *bgs13* extraction (with an abundance ratio of 4.510). This high amount of alcohol oxidase suggests possible cell lysis from the DTT extraction. A variety of vacuolar proteases that contain SP sequences, indicating they were destined for the secretory pathway, were also found. For recombinant protein expression in this strain, the presence of proteases in the cell wall and their possible effects on proteins intended for secretion begs for further consideration.

Previously characterized cell-wall associated proteins that were identified showed no general trend of down-regulation nor up-regulation regarding their relative abundance between *bgs13* and wild-type cells (Table 4); while some were found to be less abundant in the *bgs13* extraction, others were found higher in abundance, such as two 1,3-ß-glucanosyltransferase isoforms, one encoded by *GAS1* (and the other, encoded by ATY40_BA7501626), was also found in the ECM of both strains with an abundance ratio of 4.268). These enzymes, specifically

Gas1p, are involved in cell wall assembly; as shown in *S. cerevisiae*, they essentially elongate β-(1,3)-glucan chains for assembly and morphogenesis of the cell wall (Ragni et al., 2007). Interestingly, most of the proteins involved in cell wall assembly or biosynthesis were found to be lower in abundance in the *bgs13* cells than in the wild-type. The reason for this is unclear. In *S. cerevisiae*, it has been suggested that the *PKC1* kinase cascade negatively regulates some enzymes involved in the biosynthesis and assembly of the cell wall, including the gene product of *BGL2* (Shimizu et al., 1994). It is possible that a similar scenario is occurring in the *bgs13* strain of *P. pastoris*; the hybrid Remi-bgs13p may be enhancing this regulation of cell wall-associated proteins. This can be related to the difference in localization of the Remi-bgs13p, the loss of the native N-terminal domains, or due to another unknown effect of the mutation in the *BGS13* gene. Nonetheless, the higher abundance of the extracted proteins in the *bgs13* strain alluded to structural differences in the cell wall. We aimed to look at another facet of the cell wall architecture: the cell wall porosity.

Cell Wall Porosity

An initial hypothesis to explain the increase in the number of proteins exported to the extracellular media is that the cell wall of the *bgs13* cells is more porous (i.e. permeable) than that of the wild-type strain. Therefore, we performed a porosity assay on wild-type and *bgs13* strains expressing the SLPI-myc-6xHis report protein under the *AOX1* promoter. Surprisingly, the polycationic assay revealed that the *bgs13* cell walls were less porous than the wild-type cell walls. It is possible that this is related to the thicker cell wall of the *bgs13* strain (Naranjo et al., 2019) a thicker cell wall contains more cell wall constituents and possibly more proteins, leading to a lower porosity that would decrease the ingress of the polycationic polymers through the cell. The *bgs13* strain has shown increased sensitivity to Congo red and Calcofluor white, two drugs

that affect cell assembly via binding to chitin (Ram and Kliss, 2006; Naranjo et al., 2019) the thicker cell wall may be related to an elevated composition of chitin in the cell wall. However, further structural characterization of the *bgs13* cell wall constituents is needed to illuminate this aspect of its cell wall architecture. The assay suggested that a change in cell wall porosity is likely not the cause of the increased export of proteins into the extracellular media of the *bgs13* strain. However, the possibility that the change in porosity arose as an effect of the *bgs13* mutation should still be taken into consideration.

Unfolded Protein Response: Intracellular PDI Expression

In order to develop a comprehensive understanding of the mechanisms underlying the observed enhanced secretion of the *bgs13* strain, we explored what effects were occurring on the UPR after the induction of recombinant protein expression. Because Pdip and Kar2/Bip were found to be higher in abundance in the *bgs13* cell wall and ECM than the wild-type, intracellular levels of Pdip were analyzed to determine ER chaperone's abundance along its route through the secretory pathway of the mutant strain: from the ER to the ECM. Like past studies, intracellular Pdip was found in the insoluble membrane-associated fraction and not the soluble cytoplasmic fraction (Damasceno et al., 2007). The extraction of Pdip was performed on strains that recombinantly expressed the SLPI protein, whose native conformation contains eight disulfide bonds. The western analysis of the Pdip-containing insoluble membrane-associated fractions detected approximately equal amounts of the protein between the *bgs13* and wild-type strains. Taken together, the results that demonstrated higher Pdip levels in the cell wall and ECM, but similar levels inside the cell, suggested that the *bgs13* cells cannot retain the chaperone within the ER. Thus, the overexpressed Pdip gets shuttled through the secretory pathway to the cell wall and ECM. Since past research has identified UPR-associated chaperones traversing through

the secretory pathway with proteins intended for secretion (Roth et al., 2018), it is unclear whether the increased presence of the ER-resident chaperones in the *bgs13* ECM and cell wall indicates that they are a hindrance or benefit to the secretion of recombinant proteins. Thus, further analysis and characterization of the folding capabilities of the *bgs13* must be performed.

The link between quality control mechanisms of the UPR and cell wall integrity has been established in *S. cerevisiae* (Scrimale et al., 2009). Therefore, a similar situation could be taking place in the *P. pastoris* strains, leading to two possible models. Both models rely on the premise that *P. pastoris* only secrete proteins that are properly folded. In the first model, if the proteins, found in the ECM and cell wall extract of the *bgs13* strain compared to the wild-type, are in higher abundance but irregularly folded, it would suggest a type of deactivation of the UPR. In this case, misfolded and/or incorrectly modified proteins would circumvent targeted degradation by the ER-associated degradation (ERAD) pathway or by the vacuole and instead enter the cell wall or ECM. In our second model, the *bgs13* strain provides a stimulation of one aspect of the UPR, which would trigger an increase in folding with high fidelity that leads to a rise in the secretion of properly folded proteins. In our lab, structural characterization was performed on SLPI expressed under the effects of Pdip overexpression in the past (Li et al., 2010), so a similar analysis would prove useful to study the effect of the *bgs13* mutation on the expression of SLPI and its tertiary structure. To investigate this, future work would include structural characterization of the recombinant SLPI protein, produced in strains containing either the wild-type Bgs13 or Remi-bgs13 peptide, to detect any differences in the formation of the intramolecular disulfide bridges. Structural characterization can be performed via methods such as mass spectrometry and x-ray crystallography. Any elucidated differences in conformation would provide insight into the folding capabilities and UPR response of the *bgs13* strain

compared to wild-type. Regarding cell wall integrity and structure, another protein of interest for structural characterization would include the 1,3-ß-glucanosyltransferases encoded by *GAS1* because it has shown to have seven disulfide bridges, many of which are necessary for proper folding and substrate binding (Popolo et al., 2008).

Conclusion

The Remi-bgs13 peptide derived from the pREMI insertion into the *BGS13* locus led to a increased secretion of reporter proteins, indicating its potential to be a *Pichia pastoris* strain capable of universal enhanced secretion (Levin et al., 2013) of recombinant proteins. Additionally, the mutant *bgs13* allele led to an aberrant cell wall, a change in protein sorting of recombinant reporter proteins, and a different localization of the Remi-bgs13 peptide compared to the native wild-type protein (Levin et al., 2013; Moua et al., 2016; Naranjo et al., 2019). Analysis of the extracellular media and cell wall revealed an increased abundance of proteins from *bgs13,* many of which help elucidate the inner mechanisms of the *bgs13* strain involving a change in cell wall integrity and even the UPR response. Interestingly, although the *bgs13* cells produced more extractable proteins from their cell wall compared to the wild-type cells, they have a lower cell wall porosity. Thus, the increased export of proteins into the ECM is likely not due to a weaker cell wall.

Since UPR-associated proteins Kar2/Bip and protein disulfide isomerase were identified in both the ECM and cell wall in higher abundance from *bgs13,* the UPR response post-induction of recombinant expression was also examined. Although intracellular Pdip levels were found to be comparable between *bgs13* and its wild-type parent strain, higher abundance in the media and cell wall suggests that the ER-resident chaperones overwhelmed the capacity of the ER and were exported into the cell wall and subsequent extracellular media. This result points to the

possibility of an unorthodox mode of protein sorting in the *bgs13* strain (Moua et al., 2013) that causes enhanced release of proteins and a resultant aberrant cell wall.

Although mass spectrometry data revealed distinctive UPR involvement in methanol-induced recombinant expression within the *bgs13* strain, it is still unclear whether a change in the UPR response is leading to enhanced secretion of proteins with a proper conformation due to enhanced folding capabilities or increased release of misfolded proteins due to improper quality control and degradation. Thus, future experiments should aim to structurally characterize recombinantly expressed SLPI protein in order to reveal any possible differences in folding and disulfide bond formation. As another means of investigating the role of the Bgs13 peptide in the cell wall integrity pathway, we aim to identify any changes in activation of the downstream MAP kinase pathway that differ between the *bgs13* mutant and its wild-type parent. Discerning this aspect of the *bgs13* strain will reveal the true nature of its capabilities for enhanced secretion, but most importantly, it will provide insight into the secretory mechanisms of *P. pastoris* which can be utilized to optimize recombinant protein expression in this valuable host organism.

REFERENCES

Aebersold, R., Mann, M., 2003. Mass spectrometry-based proteomics 422, 10.

Anderson, N.L., Anderson, N.G., 1998. Proteome and proteomics: New technologies, new concepts, and new words. ELECTROPHORESIS 19, 1853–1861.

Baneyx, F., 1999. Recombinant protein expression in Escherichia coli. Current Opinion in Biotechnology 10, 411–421.

Baneyx, F., Mujacic, M., 2004. Recombinant protein folding and misfolding in Escherichia coli. Nature Biotechnology 22, 1399–1408.

Bossi, L., Roth, J.R., 1980. The influence of codon context on genetic code translation. Nature 286, 123–127.

Buckholz, R.G., Gleeson, M.A.G., 1991. Yeast Systems for the Commercial Production of Heterologous Proteins. Nature Biotechnology 9, 1067–1072.

Cereghino, J.L., Cregg, J.M., 2000. Heterologous protein expression in the methylotrophic yeast Pichia pastoris. FEMS Microbiol Rev 24, 45–66.

Chen, R., 2012. Bacterial expression systems for recombinant protein production: E. coli and beyond. Biotechnology Advances 30, 1102–1107.

Cherry, J.M., Hong, E.L., Amundsen, C., Balakrishnan, R., Binkley, G., Chan, E.T., Christie, K.R., Costanzo, M.C., Dwight, S.S., Engel, S.R., Fisk, D.G., Hirschman, J.E., Hitz, B.C., Karra, K., Krieger, C.J., Miyasato, S.R., Nash, R.S., Park, J., Skrzypek, M.S., Simison, M., Weng, S., Wong, E.D., 2012. Saccharomyces Genome Database: the genomics resource of budding yeast. Nucleic Acids Res 40, D700–D705.

Cregg, J.M., Madden, K.R., Barringer, K.J., Thill, G.P., Stillman, C.A., 1989. Functional characterization of the two alcohol oxidase genes from the yeast Pichia pastoris. Molecular and Cellular Biology 9, 1316–1323.

Cregg, J.M., Tschopp, J.F., Stillman, C., Siegel, R., Akong, M., Craig, W.S., Buckholz, R.G., Madden, K.R., Kellaris, P.A., Davis, G.R., Smiley, B.L., Cruze, J., Torregrossa, R., Veliçelebi, G., Thill, G.P., 1987. High–Level Expression and Efficient Assembly of Hepatitis B Surface Antigen in the Methylotrophic Yeast, Pichia Pastoris. Nature Biotechnology 5, 479–485.

Crick, F.H., Barnett, L., Brenner, S., Watts-Tobin, R.J., 1961. General nature of the genetic code for proteins. Nature 192, 1227–1232.

Delic, M., Valli, M., Graf, A.B., Pfeffer, M., Mattanovich, D., and Gasser, B., 2013. The secretory pathway: exploring yeast diversity. FEMS Microbiology Reviews 37, 872–914.

De Schutter, K., Lin, Y.-C., Tiels, P., Van Hecke, A., Glinka, S., Weber-Lehmann, J., Rouzé, P., Van de Peer, Y., Callewaert, N., 2009. Genome sequence of the recombinant protein production host Pichia pastoris. Nature Biotechnology 27, 561–566.

Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F., Whitehouse, C.M., 1989. Electrospray ionization for mass spectrometry of large biomolecules. Science 246, 64–71.

Gecchele, E., Merlin, M., Brozzetti, A., Falorni, A., Pezzotti, M., Avesani, L., 2015. A Comparative Analysis of Recombinant Protein Expression in Different Biofactories: Bacteria, Insect Cells and Plant Systems. JoVE (Journal of Visualized Experiments) e52459.

Ghaemmaghami, S., Huh, W.-K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., Weissman, J.S., 2003. Global analysis of protein expression in yeast. Nature 425, 737–741.

Hebert, A.S., Richards, A.L., Bailey, D.J., Ulbrich, A., Coughlin, E.E., Westphall, M.S., Coon, J.J., 2014. The One Hour Yeast Proteome. Mol Cell Proteomics 13, 339–347.

Heiss, S., Maurer, M., Hahn, R., Mattanovich, D., Gasser, B., 2013. Identification and deletion of the major secreted protein of Pichia pastoris. Appl Microbiol Biotechnol 97, 1241–1249.

Ho, Y., Gruhler, A., Heilbut, A., Bader, G.D., Moore, L., Adams, S.-L., Millar, A., Taylor, P., Bennett, K., Boutilier, K., Yang, L., Wolting, C., Donaldson, I., Schandorff, S., Shewnarane, J., Vo, M., Taggart, J., Goudreault, M., Muskat, B., Alfarano, C., Dewar, D., Lin, Z., Michalickova, K., Willems, A.R., Sassi, H., Nielsen, P.A., Rasmussen, K.J., Andersen, J.R., Johansen, L.E., Hansen, L.H., Jespersen, H., Podtelejnikov, A., Nielsen, E., Crawford, J., Poulsen, V., Sørensen, B.D., Matthiesen, J., Hendrickson, R.C., Gleeson, F., Pawson, T., Moran, M.F., Durocher, D., Mann, M., Hogue, C.W.V., Figeys, D., Tyers, M., 2002. Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature 415, 180–183.

Huang, C.-J., Damasceno, L.M., Anderson, K.A., Zhang, S., Old, L.J., Batt, C.A., 2011. A proteomic analysis of the Pichia pastoris secretome in methanol-induced cultures. Appl Microbiol Biotechnol 90, 235–247.

Jung, U.S., Levin, D.E., 1999. Genome-wide analysis of gene expression regulated by the yeast cell wall integrity signalling pathway. Molecular Microbiology 34, 1049–1057.

Levin, D.E., 2011. Regulation of Cell Wall Biogenesis in Saccharomyces cerevisiae: The Cell Wall Integrity Signaling Pathway. Genetics 189, 1145–1175.

Kelleher, D.J., Gilmore, R., 2006. An evolving view of the eukaryotic oligosaccharyltransferase. Glycobiology 16, 47R-62R.

Kim, Y., Nandakumar, M.P., Marten, M.R., 2007. Proteomics of filamentous fungi. Trends in Biotechnology 25, 395–400.

Kollár, R., Reinhold, B.B., Petráková, E., Yeh, H.J.C., Ashwell, G., Drgonová, J., Kapteyn, J.C., Klis, F.M., Cabib, E., 1997. Architecture of the Yeast Cell Wall β(1→6)-GLUCAN INTERCONNECTS MANNOPROTEIN, β(1→3)-GLUCAN, AND CHITIN. J. Biol. Chem. 272, 17762–17775.

Kolsrud, H., Malerod, H., Ray, S., Reubsaet, L., Lundanes, E., and Greibrokk, T., 2012. A Critical Review of Trypsin Digestion for LC-MS Based Proteomics. In Integrative Proteomics, H.-C. Leung, ed. (InTech).

Kozak, M., 1999. Initiation of translation in prokaryotes and eukaryotes. Gene 234, 187–208.

Kuehn, M.J., Herrmann, J.M., Schekman, R., 1998. COPII–cargo interactions direct protein sorting into ER-derived transport vesicles. Nature 391, 187–190.

Larsen, S., Weaver, J., de Sa Campos, K., Bulahan, R., Nguyen, J., Grove, H., Huang, A., Low, L., Tran, N., Gomez, S., et al., 2013. Mutant strains of Pichia pastoris with enhanced secretion of recombinant proteins. Biotechnol Lett 35, 1925–1935.

Levin, D.E., 2005. Cell Wall Integrity Signaling in Saccharomyces cerevisiae. Microbiol. Mol. Biol. Rev. 69, 262–291.

Li, Z., Moy, A., Gomez, S.R., Franz, A.H., Lin-Cereghino, J., Lin-Cereghino, G.P., 2010. An improved method for enhanced production and biological activity of human secretory leukocyte protease inhibitor (SLPI) in Pichia pastoris. Biochemical and Biophysical Research Communications 402, 519–524.

Li, H., Sethuraman, N., Stadheim, T.A., Zha, D., Prinz, B., Ballew, N., Bobrowicz, P., Choi, B.-K., Cook, W.J., Cukan, M., Houston-Cummings, N.R., Davidson, R., Gong, B., Hamilton, S.R., Hoopes, J.P., Jiang, Y., Kim, N., Mansfield, R., Nett, J.H., Rios, S., Strawbridge, R., Wildt, S., Gerngross, T.U., 2006. Optimization of humanized IgGs in glycoengineered Pichia pastoris. Nature Biotechnology 24, 210–215.

Lipke, P.N., Ovalle, R., 1998. Cell Wall Architecture in Yeast: New Structure and New Challenges. Journal of Bacteriology 180, 3735–3740.

Lodish, H., Berk, A., Zipursky, S.L., Matsudaira, P., Baltimore, D., Darnell, J., 2000. Overview of the Secretory Pathway. Molecular Cell Biology. 4th edition.

Jackson, C.L., 2009. Mechanisms of transport through the Golgi complex. Journal of Cell Science 122, 443–452.

Matsuura-Tokita, K., Takeuchi, M., Ichihara, A., Mikuriya, K., and Nakano, A., 2006. Live imaging of yeast Golgi cisternal maturation. Nature 441, 1007-1010.

Moua, P.S., Gonzalez, A., Oshiro, K.T., Tam, V., Li, Z.H., Chang, J., Leung, W., Yon, A., Thor, D., Venkatram, S., et al., 2016. Differential secretion pathways of proteins fused to the Escherichia coli maltose binding protein (MBP) in Pichia pastoris. Protein Expression and Purification 124, 1–9.

Naranjo, C. A., Uribe, C., Hang, A., Herr, K., Jivan, A. D., Vo, M. N., Rabara, T., Deyarmin, J. S., Fong, M. M., de Sa Campos, K., Moua, P., Myers, G., Thor, D., Risser, D. D., Vierra, C., Franz, A. H., Lin-Cereghino, J., Lin-Cereghino, G. P., 2019. Characterization of the Role of *BGS13* in the Secretory Mechanism of *Pichia pastoris*. Unpublished.

Ogata, K., Nishikawa, H., Ohsugi, M., 1969. A Yeast Capable of Utilizing Methanol. Agricultural and Biological Chemistry 33, 1519–1520.

Patil, C., and Walter, P., 2001. Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. Current Opinion in Cell Biology 13, 349–355.

Reinoso-Martín, C., Schüller, C., Schuetzer-Muehlbauer, M., Kuchler, K., 2003. The Yeast Protein Kinase C Cell Integrity Pathway Mediates Tolerance to the Antifungal Drug Caspofungin through Activation of Slt2p Mitogen-Activated Protein Kinase Signaling. Eukaryotic Cell 2, 1200–1210.

Ron, D., and Walter, P., 2007. Signal integration in the endoplasmic reticulum unfolded protein response. Nature Reviews Molecular Cell Biology 8, 519–529.

Schmitz, H.-P., Lorberg, A., Heinisch, J.J., 2002. Regulation of yeast protein kinase C activity by interaction with the small GTPase Rho1p through its amino-terminal HR1 domain: Amino-terminal regulation of yeast Pkc1p. Molecular Microbiology 44, 829–840.

Sreekrishna, K., Brankamp, R.G., Kropp, K.E., Blankenship, D.T., Tsay, J.-T., Smith, P.L., Wierschke, J.D., Subramaniam, A., Birkenberger, L.A., 1997. Strategies for optimal synthesis and secretion of heterologous proteins in the methylotrophic yeast Pichia

pastoris. Gene, Eukaryotic Expression Vector Systems: Biology and Applications 190, 55–62.

Tschopp, J.F., Brust, P.F., Cregg, J.M., Stillman, C.A., Gingeras, T.R., 1987. Expression of the lacZ gene from two methanol-regulated promoters in Pichia pastoris. Nucleic Acids Res 15, 3859–3876.

von Heijne, G., 1990. The signal peptide. J. Membrane Biol. 115, 195–201.

Walter, P., and Ron, D., 2011. The Unfolded Protein Response: From Stress Pathway to Homeostatic Regulation. Science 334, 1081–1086.

Wilkinson, B., Gilbert, H.F., 2004. Protein disulfide isomerase. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics 1699, 35–44.

Wurm, F.M., 2004. Production of recombinant protein therapeutics in cultivated mammalian cells. Nature Biotechnology 22, 1393–1398.

Yang, Z., and Zhang, Z., 2018. Engineering strategies for enhanced production of protein and bio-products in Pichia pastoris: A review. Biotechnology Advances 36, 182–195.